MILKS MEAN MORE: THE ROLE OF MILK IN NUTRITION, DIGESTION AND METABOLISM ACROSS THE LIFESPAN

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Integrating the Ecosystem Services Framework to Define Dysbiosis of the Breastfed Infant Gut: The Role of *B. infantis* and Human Milk Oligosaccharides

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Mounting evidence supports a connection between the composition of the infant gut microbiome and long-term health. In fact, aberrant microbiome compositions during key developmental windows in early life are associated with increased disease risk; therefore, making pertinent modifications to the microbiome during infancy offers significant promise to improve human health. There is growing support for integrating the concept of ecosystem services (the provision of benefits from ecosystems to humans) in linking specific microbiome functions to human well-being. This framework is widely applied in conservation efforts of macro-ecosystems and offers a systematic approach to guide restoration actions aimed to recover critical ecological functions. The aim of this work is to apply the ecosystem services framework to integrate recent studies demonstrating stable alteration of the gut microbiome of breastfed infants when *Bifidobacterium longum* subsp. *infantis* EVC001, a gut symbiont capable of efficiently utilizing human milk oligosaccharides into organic acids that are beneficial for the infant and lower intestinal pH, is reintroduced. Additionally, using examples from the literature we illustrate how the absence of *B. infantis* results in diminished ecosystem services, which may be associated with health consequences related to immune and metabolic disorders. Finally, we propose a model by which infant gut dysbiosis can be defined as a reduction in ecosystem services supplied to the host by the gut microbiome rather than merely changes in diversity or taxonomic composition. Given the increased interest in targeted microbiome modification therapies to decrease acute and chronic disease risk, the model presented here provides a framework to assess the effectiveness of such strategies from a host-centered perspective.

Keywords: gut microbiome, dysbiosis, human milk oligosaccharides, ecosystem services, microbiome modification, microbial ecology, symbiosis, probiotics

INTRODUCTION

Disruption to the composition and function of the early life gut microbiome is now recognized for its role in irregular immune development (1, 2), metabolic disorders (3) and inflammation (4, 5). Several of these phenotypes have been reconstructed using animal models or epidemiological approaches providing a compelling link between aberrant microbiome development in early life and these negative health outcomes (6–9). Thus, if pandemic non-communicable diseases such as type 1 diabetes, obesity, allergy, and asthma are associated with impaired microbiomes during infancy, as suggested by emerging evidence (3, 10–15), then relevant modulation of the microbiome in early life provides a compelling solution for addressing the increasing public health burden associated with these diseases. However, evaluative parameters to identify desirable microbiome compositions and their potential interrelationship with health, are currently lacking.

The application of methods derived from ecological theory and evolutionary biology have been fundamental to elucidating the factors that shape the microbiome throughout the lifespan. In this work, we apply concepts from the "ecosystem services" framework (16) to guide the ecological assessment of the breastfed infant gut microbiome from a host-centered perspective. We first describe the ecological processes that shape and define the composition of the microbiome in early life. This description is centered on the hypothesis that human hosts select, via diet (human milk), for the enrichment of specialized symbionts that fulfill beneficial functions underlying the provision of ecosystem services that contribute to their fitness and well-being. We then propose a model in which the absence of these beneficial functions and the consequential reduction in one or more ecosystem services can be defined as dysbiosis. To demonstrate the applicability of the model, the discussion is centered on the coevolution of specialized bifidobacteria, namely *B. infantis*, for which clinical evidence is available (17). Finally, we summarize, as evidence for this model, large cohort studies indicating the absence of bifidobacteria in early life is associated with negative health outcomes.

ECOLOGICAL PROCESSES SHAPING THE COMPOSITION OF THE BREASTFED INFANT GUT MICROBIOME

Immediately following birth, the neonatal intestine becomes rapidly colonized by microbes from the mother and the surrounding environment. Infants delivered by cesarean section are more likely to become colonized by environmental microorganisms from the maternal skin, healthcare staff and hospital surfaces. Vaginally delivered infants come in contact with bacteria from mother's vaginal canal and the fecal microbiota (18, 19). From this initial load of microbes, the allochthonous, vaginally-derived and environmental species are then rapidly replaced by organisms adapted to the gut (20–23); however, the microbiome differences based on delivery mode persist over time (19). Nutritional resources that reach the gut are another major factor influencing the neonatal gut microbiome, in terms of both composition and function. In exclusively breastfed infants, human milk oligosaccharides (HMOs) represent the main nutritional resources for bacteria in the gut. As a result, the gut microbiome of exclusively breastfed infants exhibits lower alpha diversity and higher abundance of specialized taxa able to metabolize HMOs, namely bifidobacteria (24–27). In the absence of specialized infant-associated species of bifidobacteria, HMOs are under-utilized, resulting in excess resources with profound impacts on ecosystem function (**Figure 1**). Cessation of breastfeeding and the introduction of solid foods represent a major shift in the nutritional resource landscape and a more functionally complex community of microbes is then required to deplete the greater variety of dietary substrates reaching the large intestine (**Figure 1**).

Additional ecological events, including random processes, ultimately influence the overall composition of the infant gut microbiome; however, initial microbiome inoculation based on birth mode, and the subsequent environmental selection through the provision of selective substrates from human milk, are the two major ecological processes shaping the gut microbiome of breastfed infants (30–33).

BIOLOGICAL CONSIDERATIONS IN DEFINING A HEALTHY INFANT GUT MICROBIOME

Identifying a healthy gut microbiome in both infants and adults has proven to be a major challenge to the scientific and medical fields (34). Historically, diversity has been speculated to maximize functionality, in a generalization of the "insurance hypothesis" (35–38). However, diversity indices are of limited value alone and have proven insufficient to determine ecosystem functionality, or to categorize microbial ecosystems as healthy or unhealthy (37, 39) (see **Box 1** for an in-depth discussion on the limitations of diversity). Moreover, taxonomic composition can be highly variable among individuals, while functions encoded by the gut microbiome are remarkably coherent (45) and breastfed infants across different geographies develop a common microbial functional core (15, 32, 33). This implies hosts are under a strong pressure to select high-fidelity microbial partners to maintain key ecosystem functions (38), and that breast milk establishes key niches that can only be occupied by specialized taxa (46) (**Figure 1**). Furthermore, given the host and its microbiome operate as a highly interconnected and co-evolved ecosystem in which interactions among members and community characteristics are governed by the principles of community ecology, we argue the evaluation of gut microbiomes can only be successful if based on ecological and evolutionary criteria. To this end, the ecosystem services framework has been implemented to link ecological processes of macro-ecosystems with elements of human well-being (47) and has recently been adapted to value the services of gut microbial ecosystems from a host-centered perspective (16, 48, **Box 2**). Therefore, we propose to use this framework to guide the assessment of the infant gut

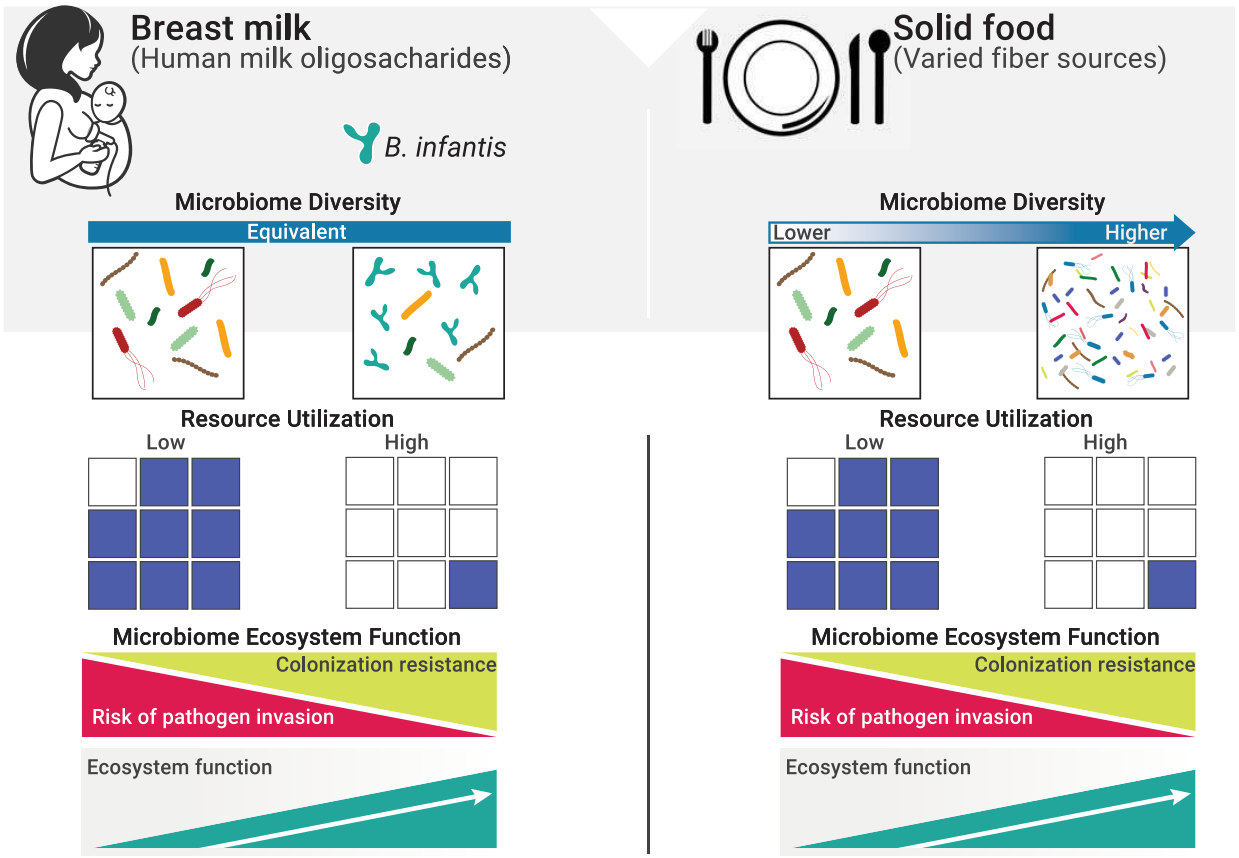


FIGURE 1 | Resource utilization and diversity in the gut in determining invasion resistance. The resource landscape in the human gut is vastly influenced by diet. One fundamental function of the microbiome is to keep potential pathogens at bay by direct competition for space and resources (i.e., co-lonization resistance, a regulating service). Excess resources (blue squares) represent open niche opportunities and increase the risk of colonization by invasive species, including pathogens. When resources are efficiently utilized, the risk of successful invasion is greatly reduced due to the lack of available resources to sustain growth (28). On a solid food diet, a more diverse composite of species is required to deplete the greater variety of resources reaching the gut (29). However, in early life, and while diet is restricted to mother's milk, resource utilization is independent from diversity and will only reach maximum levels when specialized species able to efficiently consume HMOs are present.

microbiome and to determine the ecological conditions within the gut that may increase host health and, ultimately, fitness.

In the following sections we outline three key ecosystem services: (1) supporting services; (2) provisioning services; and (3) regulating services underlying the relationship between the breastfed infant and the gut microbiome based on previously defined criteria (16). Specifically, we discuss the ability of *B. infantis* to efficiently utilize resources (i.e., HMOs) and produce organic acids as key functional traits that sustain the provision of these services.

SUPPORTING AND PROVISIONING SERVICES OF THE GUT MICROBIOME IN EARLY LIFE

Organic acids, including short chain fatty acids (SCFA), are the major metabolic products of anaerobic microbial fermentation in the gut and have demonstrated roles in human health (63, 64). In the breastfed infant gut, fermentation of HMOs into lactate and acetate depends critically on specialized primary degrader organisms that have the metabolic machinery to capture and metabolize these complex compounds (46). This process generates pioneer products (supporting service; **Table 1**) and releases energy that is otherwise inaccessible to the infant (provisioning service; **Table 1**). Selected strains of bifidobacteria and *Bacteroides* metabolize HMOs (24), but only *B. infantis* contains complete pathways enabling intracellular HMO-transport and degradation. Consequently, it is the only organism with the demonstrated capacity to significantly increase the production of lactate and acetate in the breastfed infant gut while simultaneously decreasing residual HMOs in the stool of breastfed infants (17, 65). In fact, in the absence of *B. infantis*, high concentrations of these HMOs are expelled into the stools of infants (1, 17, 66–68) which is a clear indication of low utilization of these resources (i.e., HMOs) in the gut, even when compared to infants colonized by other bifidobacteria. This observation highlights the importance of *B. infantis* in providing

BOX 1 | Diversity: How is it measured and what does it mean?

There are two main types of diversity computed in microbial ecology studies, particularly as it pertains to microbiome profiling: alpha diversity and beta diversity.

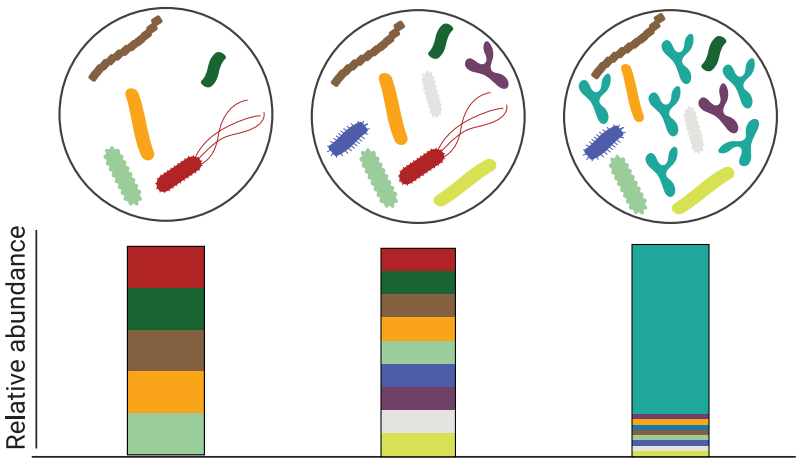
Alpha diversity refers to the measure of diversity within a specific ecological community or locality in a given sample. Depending on the metric used, this index describes either species richness (i.e., the number of different species in a community); or both species richness and the evenness (i.e., the distribution of the species' abundances in the community) (40). There are several metrics to determine alpha diversity, each different in their sensitivity to richness and evenness (41). Depending on the index used, it is possible that no change in alpha diversity may be detected despite the presence of highly divergent community compositions (**Figure 2**).
Beta diversity is a measure of diversity between samples. It answers the questions: How different is the microbial composition in one sample or group of samples compared to others? How many species are shared between samples? Similar to alpha diversity, there are different metrics to establish beta diversity. Some methods are purely qualitative based on presence/absence of species, while others include a quantitative component and take into account a phylogenetic distance between species. Each method presents its own inherent biases and sensitivity capturing changes in community composition.

Uses and limitations of diversity in microbial ecology

Diversity is speculated to maximize the functionality in a generalization of the "insurance hypothesis" (35, 36), which suggests that stabilization of communities against decline in function is improved by increasing diversity (42, 43). Thus, higher diversity is often assumed to be desirable. However, unless substantial functional redundancy exists in a microbial community, any loss in key functional species will likely alter the capacity of the microbiome to support ecosystem services (44). Further, a reduction in diversity is not necessarily unfavorable to the host, especially when it is a consequence of the selective enrichment of health-promoting symbionts.

Another inherent challenge exists in the lack of an accepted, absolute value of diversity for a given community. Moreover, as previously discussed [see (39) for an excellent discussion on the matter], diversity is relative and always constrained by method of measurement. In fact, different indices vary in their sensitivity to species richness and evenness, and inferences made can differ widely depending on the measure chosen. Thus, caution must be exercised when drawing conclusions from any one diversity index and when comparing findings across studies.

Overall, simplifying the microbiome to a measure of biodiversity has obvious limitations as it does not reflect composition or function, or relevant ecosystem properties such as stability, productivity or invisibility. We and others (37, 39) argue that the continued use of this index, without context of function, distracts the field from the development of relevant hypotheses to gain insight into the underlying ecological mechanisms driving patterns and processes in microbial communities and their potential relationship to host health.



Sample	A	B	C
Number of species (richness)	5	9	9

FIGURE 2 | Alpha diversity is independent of relative abundance. Three different bacterial communities are depicted (A–C). Corresponding relative abundance of the individual species in the bacterial comminutes is represented by the stacked bar graphs. Bacterial communities B and C have the same number of observed species ($n = 9$) but their relative abundance is different, with community C being dominated by one species. While the alpha diversity can be computed with different metrics, when accounting for community richness, communities B and C species have the same alpha diversity.

and provisioning services that underlie the overall function of the infant gut microbiome ecosystem (**Table 1**).

In addition to their role as pioneer substrates in the gut, organic acids and SCFA can enter circulation and directly affect the adipose tissue, brain, and liver (69–71). Acetate has been proposed to have an important role in inducing anti-inflammatory effects via the modulation of regulatory T-cells and anti-inflammatory cytokines (70), as well as improve mucosal epithelial integrity in the gut leading to protection from infectious disease in animal models (64). Lactate crosses the blood-brain barrier and functions as a modulator of neural activity, and is actively transported by gut epithelial cells (72–74). Acetate and lactate are also precursors of butyrate, which has anti-tumorigenic and anti-inflammatory properties and provides energy to gut epithelial cells (64). Overall, these microbially-produced organic acids have a major

BOX 2 | Advantages of an ecosystem services paradigm to evaluate the infant gut microbiome.

The application of concepts drawn from applied macroecology research has provided important insights into the mechanisms shaping the gut microbiome, especially as it relates to how microbial communities assemble, function and evolve (38, 49–53), and how these processes influence human health (54, 55).

Unlike abiotic geographies for macroecology, hosts have faced millennia of coevolution to shape the populations of microbes that colonize them. Exquisitely specific mechanisms to select for specific microbial symbionts have been described for plants (56) invertebrates (squid, insects) (57), and vertebrates (58–60). Selective pressures have shaped these interactions between host and microbe over time, and in the gut microbiome, toward selection for the key ecosystem services that improve host health (i.e., fitness). Evaluation of the infant gut microbiome through the lens of ecosystem services will facilitate the identification of key ecosystem "service providers" as those species whose functions are critical for the delivery of a given service. Colonization resistance and access to specialized foods or diets (provisioning services) are examples of ecosystem services where research may offer clues as to how services in the gut microbiome have been maximized by host-microbe interactions under strong selective pressures.

The ecosystem services framework is widely applied to evaluate terrestrial and marine ecosystems (47) and was recently adapted to evaluate the mammalian gut (16). Viewed through the lens of ecosystems services, the goods and services humans obtain from their microbiomes can be categorized as supporting, provisioning, or regulating (**Table 1**). Provisioning services are those obtained directly from the production of goods, e.g., microbial production of vitamins, antimicrobials, organic and short-chain fatty acids. Regulating services are those involved in maintaining stable ecosystem conditions, e.g., resistance to pathogen invasion. Supporting services are those necessary for the production and maintenance of all other ecosystem services, e.g., generation of pioneer products.

One main advantage of applying the ecosystem service model to evaluate the infant gut microbiome is that it facilitates the systematic identification of key "service providers" whose functional traits underpin the delivery of a given service (61, 62). By explicitly linking functional traits to ecosystem service delivery, it is possible to assign "functional importance" and "irreplaceability" indices, and correspondingly, predict the extent to which the loss of key "service provider" species can impact the ecological processes that sustain ecosystem functioning (35, 62).

TABLE 1 | Ecosystem services, functions, traits, measures, and dysfunction consequences.

Ecosystem service*	Description of benefit	Underpinning functional traits of ecosystem service providers	Measures of ecosystem's functionality	Consequences of ecosystem dysfunction
Supporting Basic ecosystem processes that maintain the generation of all other services	Generation of pioneer products (primary production)	Capacity to stably colonize and generate pioneer products efficiently from the available ecosystem resources	Bioconversion rate	Decreased ecosystem functions
Provisioning Products, nutritional compounds and energetic outputs from ecosystems	Recovery of energy from non-digestible/absorbable substrates from the host's diet	Ability to efficiently access and metabolize the available resources (i.e., HMOs)	Production of organic acids and bacterial biomass from fermentation of HMOs Residual HMOs in stool.	Inefficient resource utilization. Loss of HMOs in the stool
Regulating Moderation and maintenance of essential ecological and conditions and processes.	Resistance to invasive species and prevention of pathogenic overgrowth Maintenance of mucosal and epithelial integrity	Establish abundant and stable populations. Effectively deplete the utilize the available resources (i.e., HMOs) without cross-feeding Reduce intestinal pH through the production of organic acids	Ecosystem stability index Mucosal and epithelial barrier integrity Fecal pH	Increased vulnerability to invasion and/or to the overgrowth of virulent and antibiotic resistant gene-carrying bacteria Elevated endotoxin levels Overgrowth of mucolytic bacteria

**As outlined in by McKenney et al. (16) according to criteria established in the Millennium Ecosystem Assessment (47).*

influence on host physiology. Thus, the presence of taxa able to efficiently metabolize HMOs into these key metabolites is critical to the delivery of fundamental ecosystem services that can affect the short- and long-term health of the growing infant.

COLONIZATION RESISTANCE AND STABILITY ARE CRITICAL REGULATING SERVICES OF THE INFANT GUT MICROBIOME

One of the critical functions of the gut microbiome is to protect the immunologically naïve infant from acquiring exogenous pathogens and to prevent the overgrowth of opportunistic commensals (10, 75), a process known as colonization resistance (76, 77). Direct competition for resources, metabolic exclusion by production of organic acids, and indirect stimulation of the mucosal barrier system are well-characterized mechanisms by which the microbiome provides the host with this regulatory service (78). More competition for resources increases ecological stability at the expense of diversity by favoring the growth of specialized taxa, and limits the ability of invading microbes to establish and replicate (79). Thus, increased stability is central to the delivery of this regulatory service (**Table 1**) as stable ecosystems are inherently more resistant to external disturbances (42). In a clinical study, it was shown that colonization with *B. infantis* EVC001 significantly increases the stability of the infant microbiome (17). Moreover, consumption of HMOs by *B. infantis* produces acidic end-products mainly lactate and acetate, thereby altering the intestinal environment to prohibit the growth of pH-sensitive populations (e.g., *Enterobacteriaceae*

and *Clostridia*) (69, 80, 81) including known enteric pathogens (17, 82), many of which carry antibiotic resistant genes (83–85). Further, the resulting high abundance of bifidobacteria contributes to maintaining intestinal barrier function through the production of acetate and tryptophan metabolites, and the reduction of mucus-eroding bacteria (86–89). Thus, the regulating services infants obtain from a microbiome abundantly colonized by *B. infantis* represents an archetypal model of protection, in which the host selects (via HMOs) microbial taxa most adept at strengthening epithelial defenses as well as creating biotic (i.e., competition for resources) and abiotic (i.e., pH) resistance barriers against invasion (28). A conceptual depiction of these concepts is shown in **Figures 1**, **3**.

INTEGRATING ECOSYSTEM SERVICES IN THE EVALUATION OF THE INFANT GUT MICROBIOME

The application of traditional concepts from macroecology has proven successful in providing relevant insight into the ecological dynamics that govern the human microbiome (38, 49). According to ecological theory, ecosystem productivity can be measured by total biomass and by changes in the concentration of a limiting substrate (35, 62, 91). In the gut, dietary and host-derived carbohydrates are the primary resources for microbial metabolism (31, 32). Productivity of the ecosystem can thus be determined based on the efficiency of their utilization, and in concert, determining bacterial biomass (**Table 1**; **Figure 3**). Together, these two functions offer complementary and independent approaches to monitor productivity and to identify states in which the delivery of the ecosystem services is maximized. Thus, by combining evaluations of ecosystem productivity and the generation of ecosystem services we propose a model for the definition of dysbiosis of the breastfed infant gut as a low-functioning ecosystem, in which the gut microbiome community is characterized by (1) low stability even without perturbations (e.g., diet change or antibiotics); (2) high susceptibility to invasion by external taxa; and (3) low utilization of the available resources (i.e., HMOs). The alternative to dysbiosis or a "healthy" state is characterized as being a high-functioning ecosystem when the gut microbiome community is: (1) stable over time, (2) resistant to invasion by allochthonous bacteria; and (3) shown to exhibit a high conversion of HMOs to pioneer products and biomass of benefit to the host (**Figure 3**). Overall, by focusing on function, this model is agnostic to method and index of choice and provides a quantifiable and objective approach to evaluate the microbiome.

APPLICATION OF THE MODEL TO EVALUATE THE SYMBIOSIS BETWEEN *B. INFANTIS* AND THE BREASTFED INFANT

Humans live in symbiosis with the composite of microbial inhabitants residing in their intestinal tracts but the contribution of specific species to the overall ecosystem function and terms of the individual symbiotic relationships, which can range from commensal to parasitic, are less understood. Considering the ecosystem services the infant host obtains from selectively favoring the growth of *B. infantis,* it is evident that the symbiotic relationship is mutualistic. Free selectively consumed resources like HMOs are extremely rare in nature and the composition of HMOs is unique among mammals (92, 93). Of the thousands of species able to colonize the human gut, only a very limited number of species have the molecular machinery to utilize them (24). Within the genus bifidobacteria, only *B. infantis* encodes the complete set of genes required to transport, and intracellularly deconstruct and metabolize all the chemical structures found among HMOs (65), thus indicating maintenance of these genes is under strong selection. Indeed, since its discovery, *B. infantis* has so far been exclusively found in association with human beings (94) and phylogenetic analysis indicates humans and bifidobacteria have co-speciated (95). Taken together, the association of *B. infantis* and the breastfed infant host presents strong characteristics of an exclusive symbiotic alliance that has persisted over evolutionary timescales, whereby the human host requires the symbiont to access a significant portion of its diet (i.e., HMOs), while concurrently the symbiont benefits from the nutritional niche provided by the host. This concept is congruent with well-established models of coevolved symbioses (57, 96, 97).

Interdependent biological alliances are best understood in binary symbiotic models (57). One invariable lesson from decades of research in these model systems has been that aposymbiosis (i.e., the absence of the symbiont) can represent a major stressor to the host and often results in physiological and developmental deficiencies. For example in the well-characterized Squid-*Vibrio* model, external perturbations are markedly different between apo- and symbiotic squids (98, 99). Indeed, the presence of *V. fischeri* may help modulate the host stress responses (100). Similarly, the removal of nutritional symbionts (i.e., symbiotic bacteria that help their animal partners digest, absorb, and metabolize complex nutrients) is known to pose appreciable fitness costs to the host (96, 101, 102). Notably, the removal of vertically transmitted (from parent to offspring) nutritional symbionts has been shown to have the greatest negative impact on host fitness (102), which bears surprising parallels to the conspicuous depletion of *B. infantis* among infants with severe acute malnutrition (103) and with the inverse correlation between fecal pH and stunting (104). Further examples include aposymbiotic pea aphids which have reduced growth rates, attain a lower adult size, and are reproductively sterile (101) and fruit flies, for which the presence of the facultative symbiont *Lactobacillus plantarum* is critical to the growth and maturation of larvae ingesting nutritionally suboptimal diets (9). Together, these examples demonstrate broadly that the disruption of ancient symbiotic associations can have negative implications on the host.

All data indicate human infants have evolved to partner with key symbiotic gut bacteria specialized in metabolizing host-provided resources in the form of HMOs; however, it appears over time the role of *B. infantis* and the impact of its absence from the infant gut have become obscured, likely because the generational loss of *B. infantis* predates the advent

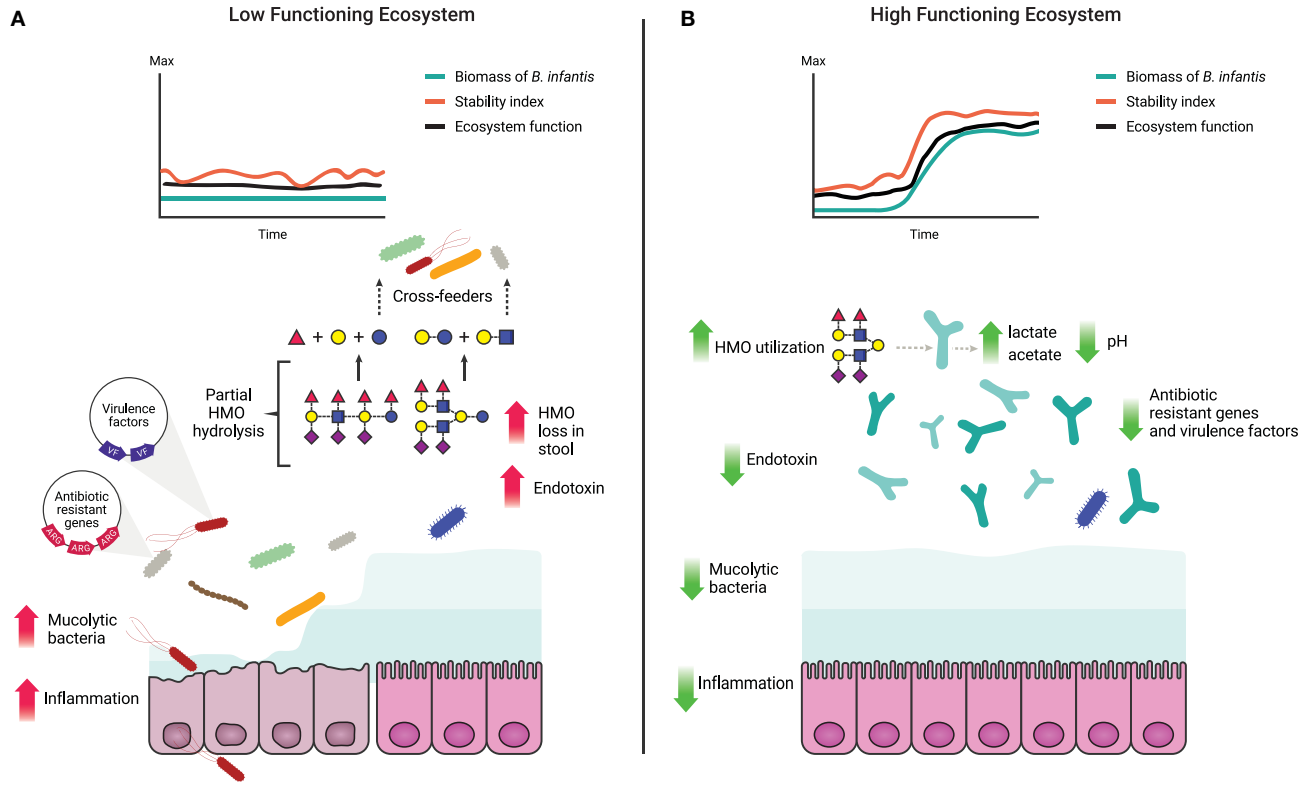


FIGURE 3 | Characteristics of a high and a low functioning infant gut microbiome based on delivery of ecosystem services. **(A)** A low functioning infant gut microbiome ecosystem where the resources (HMOs) are inefficiently utilized leading to their loss in the stool (17) and potential cross-feeding to non-adapted opportunistic taxa. Prevalence of non-adapted opportunistic taxa leads to loss of ecosystem stability and decreased ecosystem function along with increased abundance of virulence factors and antibiotic resistance genes (82, 84, 85). Overgrowth of mucus-degrading bacteria and elevated levels of endotoxin compromise the intestinal barrier leading to chronic enteric inflammation and/or increased susceptibility to bacterial translocation (5, 89, 90). **(B)** A high functioning gut microbiome where ecosystem functions and stability are maintained over time, corollary to an increase in biomass of *B. infantis*. HMOs are efficiently utilized by *B. infantis* and converted into cell biomass, organic and SCFA. Production of organic and SCFA reduces the luminal pH (17) creating an unfavorable environment for opportunistic taxa, including virulent, antibiotic resistant, and mucolytic bacteria.

of high resolution tools to investigate the gut microbiome. For instance, substantial fecal excretion of HMOs and high fecal pH are not considered abnormal, and considerable instability of the gut microbial ecosystem is considered normal in early life (33). However, historical records suggest bifidobacteria was once more prevalent among infant populations in developed nations than what contemporary reports indicate (105), and correlative evidence from large cohort studies suggest absence of this key symbiont comes with important negative acute and chronic health consequences during a critical developmental stage (2, 4, 103, 104, 106).

WHAT ARE THE ACUTE AND CHRONIC HEALTH CONSEQUENCES OF THE ABSENCE OF *B. INFANTIS* IN THE INFANT GUT?

The importance of individual species to ecosystem function, and ultimately to the services, can become apparent through their loss. There is growing appreciation that interventions known to disrupt microbiome development may lead to the extinction of certain taxa across entire populations (107). Widespread antibiotic use, cesarean section delivery, and formula feeding are associated with altered gut microbiome compositions and subsequent negative health outcomes, including obesity and autoimmune diseases (3, 108, 109). In particular, the increased prevalence of these dietary and medical interventions has been associated with the decline of *Bifidobacterium* over the past century (20, 21, 60, 105, 110, 111). We pose the loss of critical functions in the gut resulting from the decline in the prevalence of *B. infantis* may have selected for microbiota that lack the resilience and stability during critical stages of immune and metabolic development. In fact, lower abundance of bifidobacteria has been associated with greater risk for developing colic, atopic dermatitis, asthma, food allergies, type I diabetes and chronic inflammation (2, 10, 11, 15, 112). Additionally, infants lacking *B. infantis* show signs of chronic enteric inflammation during the first 60 days of life (5), which has been directly linked to an increased risk of certain chronic disorders such as atopy and asthma later in life (113).

Interestingly, in geographic locations where breastfeeding rates are high and vaginal birth is widespread, *Bifidobacterium* is normally abundant in infant microbiomes (66, 114, 115). In contrast, the gut communities of infants in developed countries are largely unstable and highly variable (25, 111) and the distribution of *Bifidobacterium* is notably bimodal (26). This variation is clearly evident in a recent comparison of the gut microbiome of infants in geographically similar but developmentally diverse locations in which the level of *Bifidobacterium* was found to be higher in infants in more resource-limited locations, which correlated with decreased incidence of autoimmune and allergic diseases (2). Together these findings raise the question of whether the modern infant gut microbiome has been fundamentally altered from that of our ancestors and how the loss of key symbiotic species and the resulting disruption in immune development could be connected to the increased incidence of metabolic, autoimmune, and allergic diseases observed in developed countries today.

Fecal pH is another factor that has changed significantly over the past century and is consistent with the loss of *Bifidobacterium* (105). Fecal pH values directly correlates with the bacterial species colonizing the infant gut, particularly pertaining is the direct association between lower fecal pH and significantly decreased abundance of potentially harmful bacterial populations (i.e., *Clostridiaceae*, *Enterobacteriaceae*, *Peptosteptoccocaceae*, and *Veillonellaceae*) (105). These findings are intriguing, as an abundance of specific *Enterobacteriaceae* species induce gut inflammation (21), which has been positively associated with colic and crying in infants (116, 117). These adverse conditions may be due to the fact that *Enterobacteriaceae*-derived lipopolysaccharides induce stronger inflammatory activity compared with other lipopolysaccharide-producing bacteria (2, 118). In addition, lower fecal pH has been shown to be associated with better anthropometric growth scores (104) and improved thymic growth, a sign of immune system development (1). This may partially explain why *B. infantis*-colonized infants exhibit more robust vaccine responses (66, 119) and why there is a reduced incidence of autoimmune diseases in populations colonized with high levels of *Bifidobacterium* (2). Taken together, these data indicate functions provided by key symbiotic partners (i.e., *B. infantis*) during infancy have a strong impact on development and conversely, the absence of these taxa can have negative health consequences, underscoring the need to restore specific beneficial taxa to the infant gut.

RELEVANCE, APPLICATIONS, AND LIMITATIONS OF THE MODEL

With the growing recognition of the role of the microbiome in human health, the incorporation of microbiome-based diagnostics will inevitably become routine. In fact, a number of commercial tests are currently available to the general public and physicians are increasingly requested to interpret test reports. However, we currently lack a "gold standard" for what constitutes a healthy microbiome. Here, we proposed an anthropocentric model whereby gut microbiome function is determined in terms of ecosystem services that ultimately benefit the infant. Thus, microbiome composition can be evaluated objectively with regard to its contribution to host health, facilitating interpretation by health professionals. Furthermore, linking functional traits to specific ecosystem services may assist both the development of prognostic tools of infant microbiome function and probiotic interventions aimed at restoring the ecosystem services of the infant gut microbiome.

However, it is important to recognize that this model is limited to conditions in which the nutrient landscape in the gut is shaped by a single nutritional resource (i.e., HMOs) and will have to be re-validated for conditions known to shift the type and amount of resources as well as the distribution of biomass. Such conditions include, the introduction of complementary foods, formula feeding, antibiotic use and other microbiome-modifying practices. Moreover, the principles on which this model is based may be affected by stochastic events including niche pre-emption (i.e., "first come, first served") driven by priority effects (120). We also recognize the overall dynamics of the infant microbiome involve complex intra and inter-species interactions which are not considered in our model. For example, the ecological relevance of *Bifidobacterium* species other than *B. infantis*, which are known to have limited capacity to metabolize HMOs but are found in the stools of infants, is currently unknown (121). Additionally, future models should aim to integrate the non-bacterial microbial inhabitants of the microbiome (e.g., virus, archaea, fungi, and other eukaryotes) which are increasingly recognized as important functional components. Nevertheless, the ecological principles presented here, can be broadly applicable to other host species, and evaluation of additional body sites, and can be adapted to inform the selection of taxa that may be relevant for health in other stages of life.

Lastly, we hope this work encourages the field to propose analogous models that incorporate ecological theory and testable frameworks to identify microbiome characteristics that are conducive to health or disease.

AUTHOR CONTRIBUTIONS

RD, BH, GC, and SF drafted and wrote this manuscript. All authors are responsible for idea conception, critical evaluation, and manuscript review.

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Conflict of Interest: RD, BH, GC, and SF are employed by Evolve BioSystems, Inc.

GLOSSARY

Allochthonous: An organism whose origin is different than that in which is found.

Biomass: Total quantity of living organisms of a species per unit area of a given habitat.

Dysbiosis: Vague/imprecise term often used to describe a microbiome composition that is different from the control and/or observed in subjects with a particular disease or condition. No scientific consensus has been reached on its definition. The origins and use of the term are reviewed by Hooks and O'Malley (122), and the casual obfuscation of its use to progress in the field, is discussed in Olesen and Alm (123) and Brussow (124).

Ecosystem services: Refers to the benefits to be gained from properly functioning ecosystems.

Ecosystem functioning: Refers to all processing and transport of energy and matter in an ecosystem, integrating multiple individual functions of the ecosystem including the production of biomass, the biochemical cycling of resources and the ability to resist invasion by allochthonous species.

Fitness (ecology): An organism's adaptation to the environment that increases its ability to propagate its genes. Genotypes with higher fitness are therefore selected for in the next generation (see natural selection). Fitness is environment-specific and directly related to the number of offspring produced.

Insurance hypothesis: Suggests that stabilization of communities against decline in function resulting from invasion, species loss, or fluctuations in abiotic features of the environment is improved by increasing diversity, and that diminishing fluctuations over time increases the overall productivity or services provided by the community.

Limiting substrate: Specific resource by which the productivity rate of an ecosystem depends on.

Mutualistic: Exchange of goods and services between species.

Niche (Hutchinsonian): Environmental conditions (biotic and abiotic) of a given habitat under which a species can persist and maintain stable populations without immigration from external sources.

Opportunistic commensal: Otherwise non-harming members of the gut microbiome that bloom upon a disturbance to the ecosystem and exert pathogenicity to the host.

Primary production: First level of nutrient generation in the tropic chain (food web).

Productivity: Rate at which energy is converted to biomass.

Stability (ecology): Measure of the temporal variability of an ecosystem, depends on its resistance to environmental change, and its rate of return to equilibrium following a perturbation (resilience).

Stochastic: With inherent randomness, the opposite of deterministic.

Symbiosis (From Greek: *sym* "with" and *biosis* "living") long-term associations between organisms of distinct genetic makeup.

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ORIGINAL RESEARCH
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In vitro Fermentation of Digested Milk Fat Globule Membrane From Ruminant Milk Modulates Piglet Ileal and Caecal Microbiota

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Lipids in milk are secreted as a triacylglycerol core surrounded by a trilayer membrane, the milk fat globule membrane (MFGM). This membrane, known to have important roles in infant brain and intestinal development, is composed of proteins, glycoproteins, and complex lipids. We hypothesized that some of the beneficial properties of MFGM are due to its effects on the gastrointestinal microbiota. This study aimed to determine the effect of a commercial phospholipid concentrate (PC) and enriched bovine, caprine, and ovine MFGM fractions on ileal and hindgut microbiota *in vitro*. Digestion of PC and MFGMs was conducted using an *in vitro* model based on infant gastric and small intestine conditions. The recovered material was then *in vitro* fermented with ileal and caecal inocula prepared from five piglets fed a commercial formula for 20 days before ileal and caecal digesta were collected. After each fermentation, samples were collected to determine organic acid production and microbiota composition using 16S rRNA sequencing. All substrates, except PC (5%), were primarily fermented by the ileal microbiota (8–14%) ($P < 0.05$). PC and caprine MFGM reduced ileal microbiota alpha diversity compared to ileal inoculum. Caprine MFGM increased and PC reduced the ileal ratio of firmicutes:proteobacteria ($P < 0.05$), respectively, compared to the ileal inoculum. Bovine and ovine MFGMs increased ileal production of acetic, butyric, and caproic acids compared to other substrates and reduced the proportions of ileal proteobacteria ($P < 0.0001$). There was a limited fermentation of bovine (3%), caprine (2%), and ovine (2%) MFGMs by the caecal microbiota compared to PC (14%). In general, PC and all MFGMs had a reduced effect on caecal microbiota at a phylum level although MFG source-specific effects were observed at the genus level. These indicate that the main effects of the MFGM in the intestinal microbial population appears to occur in the ileum.

Keywords: milk fat globule membrane, ileal and caecal fermentation, caprine, bovine, ovine

INTRODUCTION

Milk fat is secreted from the mammary gland in the form of milk fat globules (MFG) composed of a triacylglycerol core covered by a trilayer membrane, the milk fat globule membrane (MFGM). The MFGM is a source of bioactive proteins, glycoproteins, and complex lipids known to improve body (1), brain (1–4), immune (5), and intestinal development (6, 7).

MFG are digested and absorbed in different areas of the intestinal tract, which affects the nutritional and functional role of the MFGM components. Initially, it was thought that MFGM components were digested and absorbed in the small intestine (8, 9). Recent studies, however, indicate that specific components of the bovine MFGM, such as glycoproteins, could reach the large intestine (10). *In vitro* digestion of MFGM components has mainly been tested using adult conditions (time, pH, and enzyme concentration) (11, 12). The infant's gastrointestinal tract is immature with suboptimal pH for digestive enzymes and lower concentrations of bile acids and enzymes (13, 14). This could reduce the digestion of MFGM components in the stomach and small intestine and, therefore, increase the amount of undigested MFGM reaching the large intestine.

Digestion of MFGM is affected by the MFGM structure and composition (15). Human and ruminant MFGMs share the same structure, but their different protein and lipid composition may affect digestion and functionality. For instance, proteomic analysis has identified 312, 554, 175, and 140 proteins in human, bovine, caprine, and ovine MFGM, respectively, of which only 87 proteins were common among these species (16, 17). Differences in the profile of complex lipids among MFGMs have also been reported. Phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin, the three major constituents of MFGM polar lipids, account for 62–80% of the total phospholipids in human and bovine MFGM and 90% in ovine and caprine MFGMs (18–22). Thus, differences in MFGM composition may lead to different rates of digestion (15), absorption, and subsequently, different portions of the MFGM reaching the lower small intestine (ileum) prior to being released into the large intestine, where they are available for microbial fermentation.

The effects of a commercial bovine MFGM concentrate, known as phospholipid concentrate (PC), on large intestinal mucosa, microbial profile, and organic acid concentration were recently demonstrated using animal models (6, 23, 24). Bhinder et al. (6), using a "pup-in-a-cup" rat model (5–15 post-natal days of age), show that addition of PC to infant formula restored small and large intestinal growth, microbial composition, Paneth and goblet cell numbers, and tight-junction protein patterns to conditions found in pups fed maternal milk. Another study (24) shows that a combination of PC and prebiotics improved small (duodenum, jejunum, ileum) and large intestinal (ascending colon) maturation (e.g., greater enzymatic activity) and reduced opportunistic bacteria (*Escherichia/Shigella*, *Klebsiella*) in piglets compared to those fed a control formula.

Recent studies demonstrate that the ileum harbors a significant population of microbes (25) and is associated with variable retention times (72–392 min in healthy individuals) (26), which may have an important role on fermenting undigested food components prior to the large intestine (27). It has been reported, for example, that close to 30% of the digesta entering the ileum of pigs fed a humanized diet was fermented in the ileum (27). In another study, 80% of soluble kiwifruit fiber was fermented in the upper gastrointestinal tract of pigs (28), and this occurred mainly in the ileum. In human adults, a considerable degree of fiber fermentation has also been reported in the upper gastrointestinal tract [47% for pectin (29), 66% for a resistant starch (30)]. Therefore, we hypothesized that dietary bovine, caprine, and ovine MFGMs differently modulate the ileal and caecal microbiota population, resulting in different fermentation end products.

This study aimed to determine the effect of PC and enriched bovine, caprine, and ovine MFGM fractions on ileal and caecal microbiota and production of organic acids *in vitro*. *In vitro* gastrointestinal digestion of PC and MFGMs was conducted using conditions that modeled those in infants. The undigested material was then recovered and fermented *in vitro* with ileal inoculum (ileal fermentation) followed by caecal (proximal colonic fermentation) inoculum of formula-fed piglets. After each fermentation, samples were collected to determine organic acid production and microbial profile using 16S rRNA gene amplicon sequencing (**Figure 1**).

MATERIALS AND METHODS

Milk and Milk Fat Globule Membrane Enrichment Method

A commercial bovine PC product was donated by Tatua Co-operative Dairy Company Ltd., New Zealand. Tatua's PC was obtained by microfiltration of the liquid stream derived from the production of anyhydrous milk fat. Raw, non-homogenized bovine, caprine, and ovine milk were collected from local farms (Palmerston North, New Zealand) and refrigerated at 4°C until processing.

MFGM was enriched according to the method previously described (31) with some modifications. The raw milk was centrifuged at 3,500 × *g* for 15 min at 4°C and the cream layer collected. The cream layer was washed once using phosphate-buffered solution (4.3 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4, and 137 mM NaCl) followed by tri-distilled water (twice for bovine milk and only once for caprine and ovine milk). Each washing step was followed by centrifugation at 4,500 × *g* for 20 min at 4°C. Tri-distilled water was added to the cream sample (1:1 v/v) and stirred for 10 min at 50°C in a water bath. The mixture was then sheared using a kitchen mixer (Kenwood Kitchen machine, KMC 510, 1000W, Auckland, NZ), for 10 min at speed 2 to release the fat from the cream. The fat fraction was separated from the MFGM material by centrifugation at 12,000 × *g* for 30 min at 4°C. After removing the fat fraction and aqueous phase, the pellet was freeze-dried and stored at −20°C.

Digestion

MFGM and PC were digested following the *in vitro* static Infogest model (32) with modifications (pH, enzyme concentration, and time) to represent those found in 5-months-old infants (14). Simulated digestion fluids [such as simulated salivary fluids

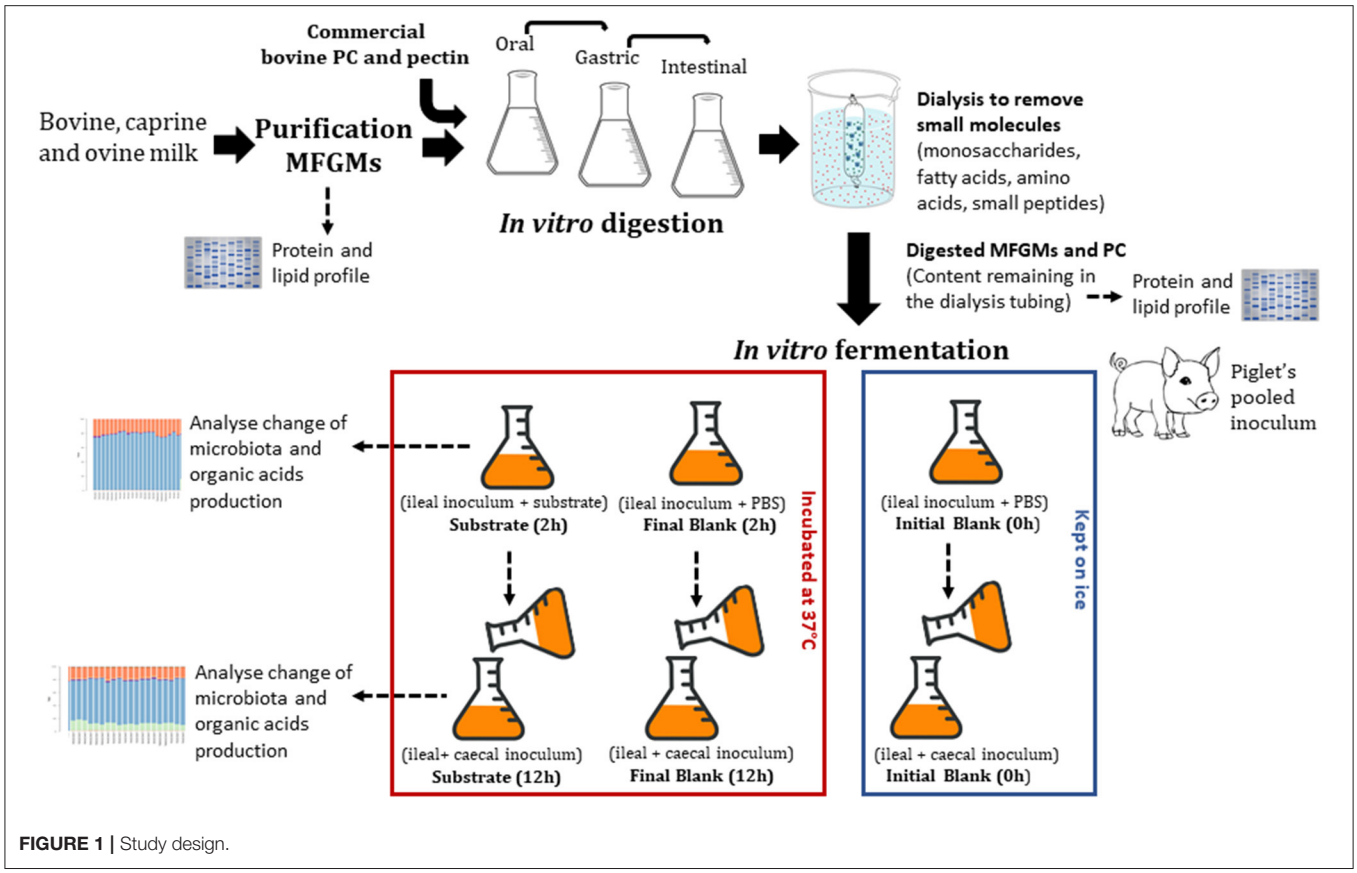

FIGURE 1 | Study design.

(SSF), simulated gastric phase solution, and small intestinal phase solution (SIF)] were made up of the corresponding electrolyte stock solutions [containing KCl, KH_2PO_4, $NaHCO_3$, NaCl, $MgCl_2(H_2O)_6$, and $(NH_4)_2CO_3$], enzymes, $CaCl_2$, and water as described in Minekus et al. (32).

Oral Phase

Dry MFGM or commercial PC treatment samples (5 g) were mixed with 3.5 mL of SSF (pH 7) (32). Salivary α-amylase solution (0.5 mL, 960 U/mL α-amylase from human saliva Type XIII-A; A1031, Sigma, St. Louis, MO, USA, made up in SSF) was added, followed by 25 μL of 0.3 M $CaCl_2$ and water to a final volume of 10 mL. All reagents were pre-warmed to 37°C before being mixed with MFGM or PC samples. Immediately after mixing, the gastric phase digestion was conducted as milk is swallowed without mastication.

Gastric Phase

The total oral digested sample was mixed with 7.5 mL of SGF (pH 6) (32), 1 mL porcine pepsin (2,000 U/mL; P6887, Sigma, made up in SGF), 1 mL gastric lipase (800 U/mL; 62305, Sigma, made up in SGF), 5 μL of 0.3 M $CaCl_2$, and water to a final volume of 20 mL. The digestion solution was incubated at 37°C for 1.5 h with the first hour at pH 6 followed by pH 5.

Intestinal Phase

The total gastric digested sample was neutralized by adjusting the pH to 6.5. The neutralized sample was then mixed with 11 mL of SIF (32), 2.5 mL of bile salts stock (16 mM; B8756, Sigma), 40 μL of 0.3 M $CaCl_2$, and water to a final volume of 40 mL. The solution was incubated for 10 min at 37°C with shaking. Pancreatin solution (5.0 mL, 800 U/mL, based on trypsin activity (P1750, Sigma, made up in SIF) was then added to the solution and incubated at 37°C for 2 h. Digestion was stopped by putting the samples on ice prior to dialysis. The small intestinal sample was then dialyzed against deionized water (Spectra, New Brunswick, USA, CE Membrane 100–500 MWCO 31 mm width, 20 mm diameter) for 24 h at 4°C. After dialysis, the samples remaining inside the dialysis tubes (i.e., undigested fraction) were freeze-dried and stored at −20°C. Dialysis is often used as a simulation model for the absorption of free amino acids and small peptides (di- or tripeptides) in the small intestine (10). After *in vitro* digestion and dialysis, the recovered material was calculated as:

$$Recovery\ material\ (\%) = \frac{((C_i - C_f)^* 100)}{C_i},$$

where C_i and C_f are the amounts of digested material added to the dialysis tube and recovered after dialysis, respectively.

Fermentation

All procedures involving animals were approved by the Grasslands Animal Ethics Committee under the New Zealand Animal Welfare Act 1999 (AEC#12997). Five male piglets 11 days old were used. During acclimatization (4 days), piglets were housed together and fed a bovine-based infant formula. Thereafter, piglets were housed individually for 16 days. The bovine-based infant formula (51.3% protein, 36.2% fat, 5.2% ash, 1.5% moisture) (Fonterra, Palmerston North, New Zealand) was provided *ad libitum* every 4 h during the whole study. On postnatal day 31 (11 days old on arrival and 20 days of study), piglets were euthanized and ileal (last 30 cm of the small intestine) and caecal digesta were collected in plastic bags previously flushed with CO_2 (to maintain anaerobic conditions) and kept on ice until fermentation (27).

The pig ileal and caecal inocula were prepared in an anaerobic cabinet. Ileal and caecal digesta were pooled (to reach the volume necessary to ferment all substrates) in similar amounts between piglets and homogenized (1:5, w/v) with a sterilized and anaerobic phosphate buffer saline solution (PBS, 0.1 M + 0.5 g/L cysteine) at pH 7 for ileum and pH 6.5 for caecum. The initial pH (pH 7 for ileum and pH 6.5 for caecum) was determined by measuring the pH in the animal ileum and caecum at the time digesta samples were collected. The pH at the end of the fermentation was not determined. Inoculum was filtered through sterilized layers of cheesecloth and stirred constantly. Ileal inoculum (5 mL) was added first to sterilized McCartney bottles containing 5 mL PBS (blank) or 5 mL PBS containing 100 mg of freeze-dried substrate (digested bovine, caprine, or ovine MFGMs, PC, or undigested citrus pectin). Citrus pectin (Spectrum Chemical, New Brunswick, USA), a highly fermentable dietary fiber, was used as a fermentation control (**Figure 1**). Pectin was not *in vitro* digested previously to the fermentation as mammalian enzymes do not cleave pectin. Thus, citrus pectin (100 mg) was fermented directly with the ileal inoculum.

Blanks (inoculum and PBS) were divided into initial and final blank. PBS was used in blanks as a high concentration of ileal and caceal digesta has been shown to provide nitrogen and minerals required by the microbiota (27). Initial blanks were kept on ice until processing, and final blanks were incubated at 37°C for 2 h for ileal or 12 h for caecal fermentation (**Figure 1**).

Twelve bottles per blank (initial and final) or per substrate were used (three technical replicates for each analysis). After 2 h of ileal fermentation, half of the bottles ($n = 6$) were removed from the incubation and kept on ice to reduce microbial activity. The remaining bottles were inoculated with the caecal inoculum using a pipette (5 mL, 1:5, w/v in PBS); initial caecal fermentation blanks were kept on ice or otherwise fermented for an additional 12 h (**Figure 1**). Half of the bottles (blanks and substrates) were placed in an autoclave (121°C for 20 min) to completely inactivate microbial fermentation and to remove the end products of organic matter fermentation (e.g., SCFA) before determination of fermentability. The other half of the bottles were thoroughly mixed, and an aliquot (1 mL) was collected into Eppendorf tubes. The Eppendorf tubes were centrifuged (14,000 × *g* for 15 min at 4°C) and the supernatant (0.5 mL) collected and stored at −20°C for subsequent determination of organic acids. The precipitate was used to determine the composition of the microbial population.

Organic matter fermentability of the MFGM substrates was calculated accordingly to Montoya et al. (33). In short,

$$\text{Organic matter fermentability}_{in\ vitro}\ (\%) = \frac{\frac{(OM-(OM_a-(OM_{blank\ initial}+OM_{blank\ final}))}{2}}{OM_b} \times 100,$$

where OM_b and OM_a are the amounts of organic dry matter in the substrate (undigested MFGM) either before or after *in vitro* fermentation. $OM_{blank\ initial}$, $OM_{blank\ final}$ are the amounts of OM in the blank bottles (which contained inoculum but no substrate) before (initial) and after (final) *in vitro* fermentation, respectively.

Chemical Composition Analysis

Proteins

Samples of MFGMs and PC before and after digestion were resuspended in water and protein concentration determined by Qubit Protein Assay (ThermoFisher, Auckland, New Zealand). The protein concentration in bovine, caprine, and ovine MFGMs and PC before and after *in vitro* digestion was adjusted to 1.6 mg/mL. The samples were further diluted (1:1) in 2× Laemmli sample buffer (5% β-mercaptoethanol) (Bio-Rad Laboratories, Inc., Hercules, CA, USA), denatured by heating at 95°C for 5 min, and centrifuged at 2,500 × *g* for 30 min at 4°C. Samples (10 μL) with a total protein concentration of 8 μg were then loaded onto SDS-PAGE gels (4–15% Mini-PROTEAN® TGX™ Precast Protein Gels, Bio-Rad Laboratories). A molecular weight (MW) marker (Precision Plus Protein Unstained Standards 3.5–260 kDa; Bio-Rad Laboratories) was used. The gels were electrophoresed in a mini-protein system (Bio-Rad Laboratories) at 110 V using a Bio-Rad power supply unit for 50 min. The SDS gels were stained for 1 h by Coomassie Brilliant Blue R-250 staining solution (Bio-Rad Laboratories) and destained overnight. Putative identification of proteins was conducted based on the estimated MW of the bands and the MW reported for each protein in previous studies for bovine (34, 35), caprine (36), and ovine (16) MFGMs. To visualize glycoproteins, other SDS-PAGE gels were loaded as described above but using a glycoprotein MW marker (CandyCane™) (ThermoFisher P21857) and a total protein concentration of 5 μg. Glycoproteins were stained using Pro-Q® Emerald 300 Glycoprotein Gel and Blot Stain Kit (ThermoFisher P21857) as per supplier instructions.

Lipid Profile

Lipid extraction and phospholipid analysis were conducted as previously described (37, 38). Total fatty acid analysis was conducted by rinsing the samples of MFGM and PC before and after digestion with 1:1 chloroform methanol and drying prior to methylation. The samples were then methylated in a two-stage process. First, 1.5 mL of 0.5 M NaOH in methanol was added to the samples and incubated for 10 min at 80°C. Second, 2.5 mL of boron trifluoride complex in methanol (10%) was

added to the samples and incubated for 30 min at 80°C. After cooling, the fatty acid fraction was extracted by adding 1 mL of iso-octane to the mixture and vortexed, followed by 5 mL of saturated NaCl, which was mixed by shaking for 30 s by hand. The sample was centrifuged (500 × *g* for 10 min) and the iso-octane layer transferred to a vial. The extraction was repeated a second time with 1 mL of iso-octane and combined with the first fraction. Samples (1 μL) were injected onto a Polar FAME (Restek RTX 2330 column, 105 m × 0.25 mm i.d, 0.20 μm film thickness; Restek Corporation, Bellefonte, USA), and fatty acid profiles were analyzed by gas chromatography (GC).

Organic Acid Detection

The preparation of samples for organic acid analysis was a two-step procedure. In the first step, organic acids were extracted into an aqueous solution for GC Flame Ionization Detection (GC-FID). GC-FID analysis of acetic, butyric, propionic, valeric, *iso*-valeric, *iso*-butyric, and caproic acids were conducted (39). The second step was an ether extraction followed by derivatization with N-methyl-N-t-butyl-dimethyl-silyl-trifluoroacetamide (Sigma-Aldrich) for GC analysis of lactic, formic, and succinic acids. These latter three organic acids have to be derivatized because formic acid responds poorly to FID, and lactic and succinic acids both have low volatility and high polarity (40). In short, 100 μL concentrated HCl and 800 μL diethyl ether were mixed vigorously with 200 μL of acidified fermented medium fluid supernatant containing the internal standard, 2-ethyl butyric acid (W242918, Sigma-Aldrich). After allowing the mixed sample to settle for 1 min, the top ether layer was transferred into a 2-mL vial. This extraction process was repeated by adding a further 800 μL diethyl ether to the aqueous phase of the first vial. The two extracts were then combined. The extract (800 μL) was derivatized with 100 μL of derivatization reagent with N-methyl-N-t-butyl-dimethyl-silyl-trifluoroacetamide and heated in a crimp-top GC vial for 20 min at 80°C. Samples were left at room temperature for ∼48 h to allow for complete derivatization of lactic acid.

The analysis was carried out using a Shimadzu GC-2010 Plus gas chromatogram (Shimadzu Corporation, Kyoto, Japan) with helium ionization detector and a Zebron ZB-5MS 30 m × 0.25 mm I.D. × 0.25 μm film capillary column. A split injection of a 1 μL sample was made at a ratio of 20:1 with a column helium flow rate of 21 mL/min. Injector and detector temperatures were both 240°C. The column temperature was initially held at 50°C for 2 min and then increased at 5°C/min to 130°C, followed by 15°C/min to 240°C (held for 4.7 min).

Microbial Profile

Microbial DNA was extracted from pellets using Machey Nagel NucleoSpin Soil kits (Macherey-Nagel, Duren, Germany) following the manufacturer's instructions with the addition of a bead-beating step using a BioSpec Mini-Beadbeater 96 set to 4 min and 0.1 mm silica beads (BioSpec, Bartlesvile, USA). DNA samples were analyzed by 16S rRNA gene amplicon sequencing using the Illumina MiSeq Platform with 2 × 250 bp paired-end sequencing. Primers targeting the V3 and V4 region of the 16S rRNA gene were used for amplification as follows: forward primer: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; reverse primer: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC.

PCR thermal cycler conditions were used as specified in the Illumina library preparation protocol (95°C for 3 min; 25 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s; 72°C for 5 min; hold at 4°C; Illumina 2015). Sequence reads were paired and quality trimmed using Qiime 1.9 (41) with the default parameters except for the following: chimeric sequence removal and OTU picking was performed using Usearch61 and taxonomy assigned against the Silva 128 small subunit ribosomal RNA database. Following quality trimming, the median number of reads was 29,295 with a minimum of 13,019, maximum of 51,579, and a standard deviation of 9,315. Alpha diversity was assessed using Faith's phylogenetic diversity indices and differences between means analyzed by ANOVA.

Statistical Analysis

Overall microbial communities were compared using permutation multivariate analysis of variation (MANOVA) using distance matrices as implemented using the adonis function in the vegan package for R (42). Comparisons of relative abundances for individual taxa were performed by permutation ANOVA using the aovp function in the lmPerm package (43) for R. *P*-values were adjusted for multiple testing using the Benjamini–Hochberg false discovery rate (FDR) method. Fisher's LSD test was used for *post-hoc* pairwise analysis.

A two-way ANOVA model was used to determine the effects of MFGMs/PC ($n = 4$), inoculum ($n = 2$), and the interaction on OM fermentability using the Proc Mixed of the statistical software SAS. For organic acid production, a one-way ANOVA model was used. The model diagnostics for each response variable were tested using the ODS graphics procedure and the repeated statement of SAS. The repeated statement was used to test for homogeneity of variances by fitting models with the restricted maximum likelihood method and comparing using the log-likelihood ratio test. The selected model for each response variable had adjusted equal variances across treatments. When the *F* value of the model was significant ($P \leq 0.05$), the means were compared using the adjusted Tukey test.

RESULTS

Milk Fat Globule Purification and Composition

The enrichment method increased the concentration of proteins putatively identified in **Figure 2** as part of the MFGM [mucin 1 (250–450 kDa), xanthine oxidase (band a), PAS III (band b), CD36 (band c), butyrophilin (band d), adipophilin and/or lactadherin (PAS 6/7; 52–58 kDa bovine, 55 kDa caprine) (band e or f), compared to bovine (lanes 2 and 3), caprine (lanes 4 and 5), and ovine lanes 6 and 7)] milk. Skim milk proteins (caseins, bands g–i) and whey proteins (β-lactoglobulin, α-lactalbumin, band j and l) were observed in all MFGM samples after enrichment. Based on band intensity, a higher proportion of caseins was observed in commercial

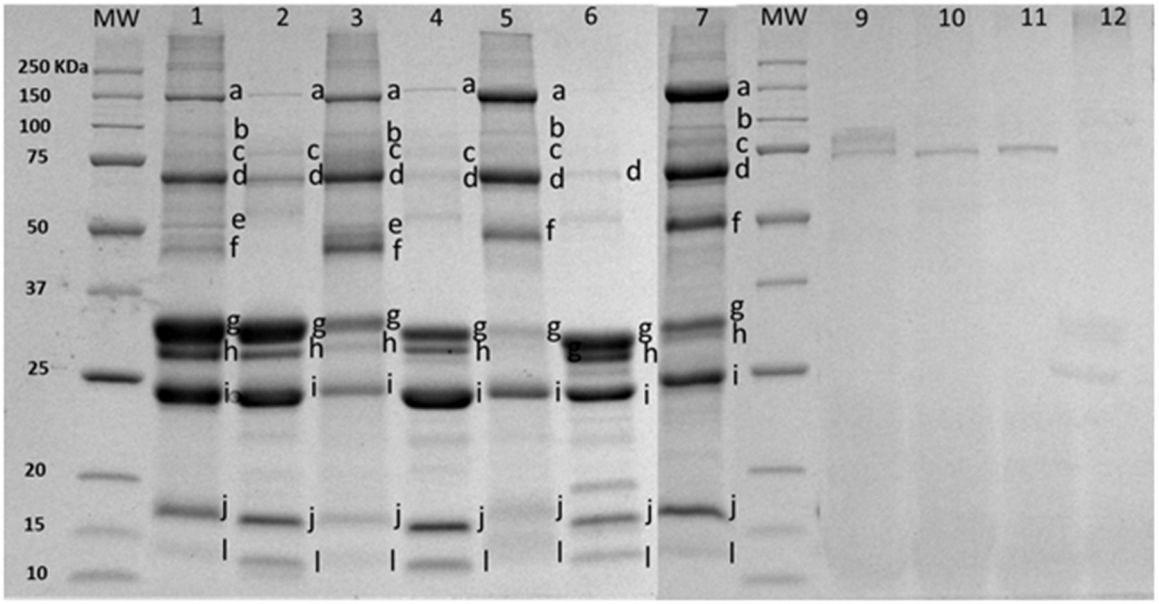

FIGURE 2 | SDS-PAGE pattern of milk fat globule membrane (MFGM) proteins in milk and MFGM purified before and after digestion. MW, molecular weight marker (KDa). The MFGM proteins are from phospholipid concentrated (lane 1), bovine milk (lane 2), bovine MFGM (lane 3), caprine milk (lane 4), caprine MFGM (lane 5), ovine milk (lane 6), ovine MFGM (lane 7), material remaining after digestion for bovine MFGM (lane 9), caprine MFGM (lane 10), ovine MFGM (lane 11), and phospholipid concentrated (line 12). Proteins were identified accordingly with MW as (a) xanthine oxidase (∼145 kDa), (b) PAS III (∼100 kDa), (c) CD36 (∼75 kDa), (d) butyrophilin (64 kDa bovine, 67 kDa caprine), (e) or (f) adipophilin (50–52 kDa) or lactadherin (PAS 6/7; 52–58 kDa bovine, 55 kDa caprine), (g) α-caseins (∼22–25Kda), (h) β-caseins (∼23–24 kDa), (i) κ-caseins (∼19 kDa), (j) β-lactaglobulin (∼18 kDa), and (l) α-lactalbumin (∼14 kDa).

TABLE 1 | Protein and lipid composition (%) of phospholipid concentrated (PC) and purified milk fat globule membrane from bovine, caprine, and ovine before and after infant simulated *in vitro* digestion.

	Before digestion				After digestion			
	PC	**Bovine**	**Caprine**	**Ovine**	**PC**	**Bovine**	**Caprine**	**Ovine**
Total protein	45.1	25.3	22.7	22.2	3.2	4.2	4.5	6.3
Total lipids	14.1	36.1	14.5	30.3	12.5	33.0	10.9	11.8
Total polar lipids	5.22	4.24	5.83	3.06	2.13	1.78	1.05	1.16
Phosphatidylinositol	0.29	0.25	0.47	0.27	0.12	0.16	0.09	0.11
Phosphatidylethanolamine	1.15	0.92	0.66	0.06	0.03	0.12	0.01	0
Phosphatidylserine	1.06	0.51	1.52	0	0	0	0	0
Phosphatidylcholine	1.69	1.52	1.92	1.29	0.24	0.48	0.13	0.2
Sphingomyelin	1.03	1.04	1.25	1.44	1.74	1.01	0.82	0.85

PC (lane 1) compared to purified bovine MFGM (lane 3). Total protein concentration in PC and purified bovine, caprine, and ovine MFGMs were 45.1, 25.3, 22.7, and 22.2%, respectively (**Table 1**).

After *in vitro* digestion and dialysis, the recovered material (content remaining in the dialysis tube) was 59, 35, 15, and 47% of the original PC, bovine, goat, and sheep MFGM, respectively. The SDS-PAGE pattern of MFGM after digestion showed only a small number of uncharacterized protein bands with a MW similar to PAS III and CD36 in bovine, ovine, and caprine MFGM samples but not in PC (**Figure 2**, lanes 10–13, respectively). The total protein in the recovered digested MFGM was 3.2, 4.2, 4.5, and 6.3% for PC, bovine, caprine, and ovine, respectively (**Table 1**).

The SDS-PAGE pattern of glycoproteins in milk, enriched MFGM samples, and PC (before and after digestion) are shown in **Figure 3**. Bands were putatively identified, based on MW, as xanthine oxidase, PAS III, CD36, butyrophilin, and lactadherin. After gastrointestinal digestion, a large proportion of glycoproteins were observed (lanes, 2, 5, 8, and 11), especially with lower MW than the parent glycoproteins observed in the intact MFGM (lanes, 1, 4, 7, and 10).

The total polar lipid content in PC, bovine, caprine, and ovine MFGM was 5.2, 4.2, 5.8, and 3.0%, respectively (**Table 1**). Phosphatidylcholine was the most concentrated phospholipid in PC (1.7%), bovine (1.5%), and caprine (1.9%) enriched MFGM and sphingomyelin was for ovine MFGM (1.4%). For all samples,

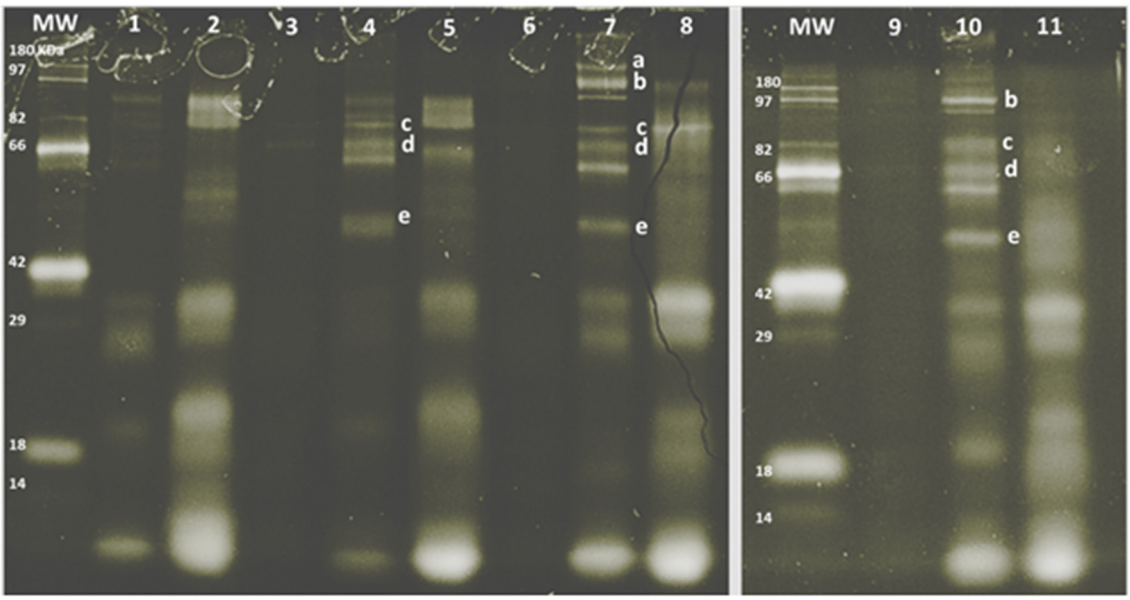

FIGURE 3 | SDS-PAGE pattern of milk fat globule membrane (MFGM) glycoproteins in milk and MFGM purified before and after digestion. MW, molecular weight marker. The MFGM proteins are from phospholipid concentrate (lane 1), digested phospholipid concentrate (lane 2), bovine milk (lane 3), bovine MFGM (lane 4), digested bovine MFGM (lane 5), caprine milk (lane 6), caprine MFGM (lane 7), digested caprine MFGM (lane 8), ovine milk (lane 9), ovine MFGM (lane 10), and digested ovine MFGM (line 11). Proteins were identified accordingly with MW as (a) xanthine oxidase (∼145 kDa), (b) PAS III (∼100 kDa), (c) CD36 (∼75 kDa), (d) butyrophilin (64 kDa bovine, 67 kDa caprine), (e) lactadherin (PAS 6/7; 52–58 kDa bovine, 55 kDa caprine).

the concentration of polar lipids was reduced after digestion to 1.0–2.1% of total lipids.

Palmitic acid (C16) and the fatty acids C18:1c9, C:18, and C14 were the most abundant fatty acids in PC (23, 29, 12, and 8%), bovine (29, 16, 17, and 11%), caprine (32, 19, 16, and 9%) and ovine (25, 20, 17, and 8%) MFGM fractions, respectively (**Table 2**). Digestion of PC, bovine, caprine, and ovine MFGM fractions had a small effect (≤10% variation) on the proportions of the fatty acids C18:2n6, C15:0, C20:3n6, C9 t11-CLA, iso C17, iso C16, C20:0, anteiso C17, C17:1, and C16:0. In contrast, a reduction of ≥65% on the proportion of the medium chain fatty acids (MCFA) (C10:0, C12:0) and C14:1 was observed after the digestion of all samples.

Ileal and Caecal Fermentation

There was a significant interaction between the location of fermentation (i.e., inoculum) and the substrate (i.e., digested MFGMs) on the organic matter fermentability ($P < 0.05$; **Table 3**). All substrates, except PC, were primarily fermented by the ileal microbiota ($P < 0.05$). As expected, pectin had the highest ileal and caecal fermentability (28 and 31%, respectively, $P < 0.05$) compared to bovine (ileal, 7.7% and caecum, 11.2%), caprine (8.8 and 10.6%), ovine (14.0 and 16.2%), MFGMs and PC (5.5 and 19.3%).

After 2 h of ileal microbiota incubation at 37°C, a decrease in the proportions of Fusobacteria was observed in the final blank compared to the initial blank (**Table 4**). At the genus level, higher unclassified *Peptostreptococcaceae* and lower proportions of *Turicibacter* and *Fusobacterium* genera were observed in the final blank compared to the initial blank (**Table 5**). Incubation of digested ovine and bovine MFGM fractions for 2 h had no effect on ileal microbial alpha diversity (**Figure 4A**) but increased the firmicutes:proteobacteria ratio (**Table 4**) compared to the initial and final blanks.

PC and caprine MFGMs reduced the ileal microbial alpha diversity compared to blanks and bovine and ovine MFGMs (**Figure 4A**). Although PC maintained the ratio of firmicutes:proteobacteria to the levels observed in the initial blank (**Table 4**), caprine MFGM increased the ratio of firmicutes:proteobacteria compared to the initial and final blanks (**Table 4**). In general, PC reduced the proportions of unclassified *Peptostreptococcaceae*, unclassified Clostridiales, *Veillonella*, *Lactobacillus*, *Prevotella*, and *Rothia* and increased the proportions of unclassified *Enterobacteriaceae*, *Turicibacter*, and *Escherichia*/*Shigella* compared to the other substrates and the final blank (**Table 5**). In general, caprine MFGM increased *Streptococcus* and decreased *Prevotella* genera compared to both blanks and other substrates. Bovine, caprine, and ovine MFGMs decreased the proportions of *Escherichia*/*Shigella* genera and unclassified *Enterobacteriaceae* compared to the final blank.

A higher concentration of acetic acid was produced by ileal microbiota fermenting ovine MFGM, whereas higher concentrations of butyric and caproic acids were produced when fermenting bovine and ovine MFGMs (**Table 6**) compared to other substrates. Formic and lactic acid production did not differ between the different types of digested MFGMs ($P > 0.05$).

Incubation of the caecal inoculum (initial blank) for 12 h reduced the alpha-diversity (final blank) (**Figure 4B**) and

TABLE 2 | Fatty acid profile (% of total fatty acids[a]) of phospholipid concentrated (PC) and purified milk fat globule membrane (MFGM) from bovine, caprine, and ovine before and after infant simulated *in vitro* digestion.

Substrate	Before digestion				After digestion			
	PC	Bovine	Caprine	Ovine	PC	Bovine	Caprine	Ovine
C10:0	1.84	2.45	5.60	4.05	0.18	0.28	0.40	0.36
C12:0	3.44	3.21	3.25	2.65	1.06	0.73	0.66	0.51
C14:0	8.65	11.16	9.34	8.31	6.28	7.71	5.47	4.54
iso C14	0.00	0.10	0.00	0.08	0.00	0.05	0.00	0.00
C14:1	0.45	0.43	0.07	0.06	0.16	0.10	0.00	0.00
C15:0	0.82	1.16	0.94	0.94	0.77	1.07	0.84	0.81
iso C15	0.16	0.33	0.14	0.21	0.15	0.25	0.11	0.15
Anteiso C15	0.34	0.46	0.20	0.34	0.28	0.33	0.13	0.22
C16:0	23.28	29.28	32.17	25.91	25.12	30.11	34.74	25.74
iso C16	0.17	0.25	0.25	0.21	0.17	0.26	0.27	0.22
C16:1	1.33	0.67	0.34	0.41	1.10	0.45	0.24	0.32
C17:0	0.44	0.67	0.83	0.83	0.51	0.73	1.02	0.93
iso C17	0.58	0.67	0.49	0.74	0.59	0.70	0.55	0.80
Anteiso C17	0.43	0.45	0.36	0.44	0.45	0.47	0.42	0.51
C17:1	0.30	0.22	0.21	0.20	0.32	0.17	0.19	0.20
C18:0	12.69	17.25	16.98	17.75	14.08	19.33	22.31	21.25
C18:1 c11	1.03	0.68	0.64	0.73	1.17	0.86	0.83	0.98
C18:1 t11	2.91	5.90	0.82	4.49	3.36	7.34	1.11	6.30
C18:1 t9	0.26	0.19	0.29	0.26	0.35	0.31	0.44	0.40
C18:1c9	28.93	16.08	19.47	20.89	32.61	20.49	23.62	27.15
C18:2 n6	2.74	0.68	2.15	1.04	2.54	0.78	1.85	1.06
C18:3 n3	0.99	0.40	0.43	0.58	0.76	0.34	0.25	0.37
C20:0	0.17	0.23	0.35	0.29	0.17	0.26	0.51	0.37
C20:1 8	0.00	0.03	0.00	0.06	0.00	0.00	0.00	0.00
C20:3n6	0.29	0.06	0.00	0.04	0.28	0.10	0.00	0.00
C20:4 n6	0.39	0.10	0.20	0.13	0.32	0.12	0.17	0.17
C20:5 n3	0.00	0.04	0.00	0.04	0.00	0.00	0.00	0.00
C22	0.22	0.19	0.20	0.15	0.25	0.26	0.28	0.23
C22:1	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00
C22:2	0.33	0.08	0.08	0.10	0.20	0.10	0.20	0.17
C22:5	0.58	0.13	0.40	0.31	0.52	0.19	0.27	0.31
C22:6 n3	0.08	0.00	0.12	0.09	0.00	0.00	0.00	0.00
C24:0	0.13	0.14	0.11	0.12	0.17	0.16	0.15	0.18
C24:1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C9 t11-CLA	1.21	0.76	0.26	1.44	1.22	0.81	0.00	1.01
MCFA	5.28	5.66	8.85	6.69	1.24	1.01	1.06	0.87
LCFA	87.35	87.52	86.67	84.98	91.52	92.32	95.07	92.48
VLCFA	2.55	1.33	1.16	2.21	2.35	1.51	0.90	1.91
SFA	53.35	67.99	71.20	63.02	50.23	62.71	67.85	56.82
UFA	41.82	26.52	25.48	30.86	44.88	32.14	29.18	38.44
MUFAs	35.21	24.27	21.84	27.10	39.05	29.71	26.43	35.35
PUFAs	5.40	1.49	3.39	2.32	4.62	1.62	2.74	2.08

MCFA, medium-chain fatty acids (C6-10). LCFA, long-chain fatty acids (C13-21). VLCFA, very long-chain fatty acids (C22 or higher). SFA, saturated fatty acids. UFA, unsaturated fatty acids. MUFAs, mono-unsaturated fatty acids. PUFAs, polyunsaturated fatty acids.

[a] *The results for the fatty acid profile analysis are in % fatty acid but do not add up to 100% as unidentified fatty acid were observed in the samples.*

changed microbial composition as observed in the principal coordinates analysis (PCoA; **Figures S1–S3**). An increased firmicutes:proteobacteria ratio was observed in the final blank compared to the initial blank (**Table 7**). At the genus level, higher unclassified *Peptostreptococcaceae*, unclassified *Lachnospiraceae, Clostridium*, *Oscillibacter*, *Turicibacter*, *Caprococcus*, and

TABLE 3 | *In vitro* ileal and caecal organic matter (OM) fermentability of digested bovine, caprine, and ovine milk fat globule membrane (MFGM); phospholipid concentrate (PC); and pectin substrates in piglets.

Substrate	PC[1]		Bovine[1]		Caprine[1]		Ovine[1]		Pectin[1]			*P*-value		
Location	Ileum	Caecum	Ileum	Caecum	Ileum	Caecum	Ileum	Caecum	Ileum	Caecum	SEM	Location (L)	Substrate (S)	L × S
OM fermentability %	5.5^{e}	19.3^{bc}	7.7^{de}	11.2^{cd}	8.8^{de}	10.6^{cde}	14.0^{cd}	16.2^{cd}	27.7^{ab}	30.5^{a}	1.8	<0.001	<0.001	0.030

[1] *Ileal fermentation (2 h) was conducted with the undigested substrates prior to caecal fermentation. Means without a common letter differ, $P < 0.05$. SEM, Standard Error of the Mean.*

TABLE 4 | Relative abundances of bacteria at the phylum level for the piglet's ileal inoculum (initial blank) and after 2 h of incubation alone (final blank) or with bovine, caprine, and ovine MFGM; phospholipid concentrate (PC); and pectin substrates.

Phyla	Initial blank[1]	Final blank[1]	PC[1]	Bovine[1]	Caprine[1]	Ovine[1]	Pectin[1]	*P*-value
Firmicutes	74.2 ± 1.3^{c}	77.5 ± 0.6^{b}	74.6 ± 0.7^{c}	79.9 ± 2.3^{ab}	80.8 ± 0.2^{a}	79.6 ± 0.9^{ab}	78.3 ± 2.7^{ab}	0.002
Proteobacteria	22.5 ± 1.1^{a}	20.2 ± 0.8^{b}	24.3 ± 0.8^{a}	17.1 ± 1.4^{c}	17.5 ± 0.2^{c}	18.0 ± 0.7^{bc}	20.1 ± 2.4^{b}	<0.001
Fusobacteria	2.3 ± 0.2^{a}	1.2 ± 0.2^{bc}	0.6 ± 0.01^{c}	1.8 ± 0.9^{ab}	0.9 ± 0.1^{c}	1.2 ± 0.1^{bc}	1.0 ± 0.3^{c}	0.006
Actinobacteria	0.31 ± 0.07^{bc}	0.37 ± 0.04^{ab}	0.15 ± 0.05^{d}	0.47 ± 0.12^{a}	0.41 ± 0.02^{ab}	0.48 ± 0.03^{a}	0.25 ± 0.09^{cd}	<0.001
Bacteroidetes	0.25 ± 0.03^{ab}	0.24 ± 0.01^{ab}	0.04 ± 0.01^{c}	0.27 ± 0.07^{a}	0.07 ± 0.006^{c}	0.20 ± 0.002^{b}	0.078 ± 0.03^{c}	<0.001
TM7	0.11 ± 0.03^{bc}	0.17 ± 0.03^{ab}	0.04 ± 0.007^{d}	0.17 ± 0.04^{ab}	0.12 ± 0.002^{bc}	0.20 ± 0.05^{a}	0.10 ± 0.04^{cd}	0.003
Firmicute:Proteobacteria	3.30 ± 0.24^{de}	3.83 ± 0.20^{cd}	3.07 ± 0.13^{e}	4.70 ± 0.51^{a}	4.62 ± 0.09^{a}	4.41 ± 0.24^{ab}	3.94 ± 0.65^{bc}	<0.001

[1] *Values are presented as mean ± standard deviation. Means within each phylum without a common letter differ, $P \leq 0.05$.*

TABLE 5 | Relative abundances of significant bacteria classified to the lowest identified taxonomic level in the piglet's ileal inoculum (initial blank) and after 2 h of incubation alone (final blank) or with bovine, caprine, and ovine MFGM; phospholipid concentrate (PC); and pectin substrates.

Taxa[1]	Initial blank[2]	Final blank[2]	PC[2]	Bovine[2]	Caprine[2]	Ovine[2]	Pectin[2]	*P*-value
Unclassified *Peptostreptococcaceae* (f)	22.6 ± 0.6^{d}	27.1 ± 0.7^{a}	21.6 ± 0.5^{d}	25.5 ± 0.1^{bc}	26 ± 0.3^{ab}	25.7 ± 0.8^{bc}	24.6 ± 0.7^{c}	<0.001
Turicibacter (g)	15.7 ± 0.8^{b}	11.9 ± 0.9^{e}	17.1 ± 0.7^{a}	12.3 ± 0.5^{e}	11.8 ± 0.2^{e}	12.6 ± 1.5^{de}	14.2 ± 0.4^{c}	<0.001
Escherichia/Shigella (g)	11.4 ± 0.7^{b}	10.7 ± 0.8^{bc}	12.9 ± 0.4^{a}	9.0 ± 0.3^{e}	9.1 ± 0^{e}	9.6 ± 0.4^{de}	10.3 ± 0.1^{cd}	<0.001
Unclassified *Enterobacteriaceae* (f)	6.8 ± 0.3^{ab}	5.9 ± 0.6^{bcd}	7.4 ± 0.1^{a}	4.6 ± 0.9^{e}	4.9 ± 0.1^{de}	5.1 ± 0.3^{de}	5.7 ± 1.2^{cd}	<0.001
Streptococcus (g)	5.2 ± 0.3^{bc}	5.9 ± 0.2^{bc}	4.8 ± 0.4^{c}	6.8 ± 0.8^{ab}	7.8 ± 0.4^{a}	5.9 ± 1.5^{bc}	5.1 ± 1.8^{bc}	0.010
Fusobacterium (g)	2.1 ± 0.2^{a}	1.0 ± 0.1^{bc}	0.6 ± 0.0^{c}	1.5 ± 0.8^{ab}	0.8 ± 0.1^{c}	1.0 ± 0.0^{bc}	0.9 ± 0.2^{c}	0.005
Enterobacter (g)	2.0 ± 0.1^{a}	1.6 ± 0.1^{ab}	2.2 ± 0^{a}	1.4 ± 0.2^{b}	1.1 ± 0^{b}	1.3 ± 0.1^{b}	2.2 ± 0.9^{a}	0.002
Unclassified Clostridiales (o)	1.8 ± 0.2^{abc}	2.0 ± 0.1^{a}	1.2 ± 0.1^{d}	2.0 ± 0.3^{ab}	1.6 ± 0^{bc}	1.8 ± 0.2^{abc}	1.6 ± 0.2^{c}	0.005
Veillonella (g)	0.4 ± 0^{ab}	0.5 ± 0^{a}	0.2 ± 0^{c}	0.3 ± 0.1^{bc}	0.4 ± 0^{bc}	0.5 ± 0^{ab}	0.3 ± 0.1^{bc}	0.004
Lactobacillus (g)	0.4 ± 0.1^{bc}	0.5 ± 0.1^{ab}	0.3 ± 0^{c}	0.5 ± 0.1^{ab}	0.6 ± 0^{a}	0.5 ± 0^{ab}	0.4 ± 0.1^{abc}	0.050
Prevotella (g)	0.09 ± 0.02^{a}	0.09 ± 0.01^{a}	0.01 ± 0.01^{b}	0.09 ± 0.04^{a}	0.02 ± 0.01^{b}	0.07 ± 0.01^{a}	0.02 ± 0.01^{b}	0.001
Rothia (g)	0.24 ± 0.05^{bc}	0.27 ± 0.01^{abc}	0.12 ± 0.05^{d}	0.36 ± 0.08^{a}	0.32 ± 0.03^{ab}	0.36 ± 0.02^{a}	0.20 ± 0.09^{cd}	<0.001

[1] *Taxa identified to the lowest rank; (g) genus, (f) family, (c) class, (o) order.*

[2] *Values are presented as mean ± standard deviation. Means within each bacterial taxon without a common letter differ, $P \leq 0.05$.*

unclassified Clostridia genera and lower proportions of *Escherichia/Shigella*, unclassified Ruminococcaceae, *Prevotella*, *Megasphaera*, *Bacteroides,* unclassified Prevotellaceae, and *Fusobacterium* genera were observed in the final blank compared to the initial blank (**Table 8**).

In general, after 12 h of incubation with caecal inoculum, PC and all MFGMs had no effect of caecal microbiota diversity (**Figure 4B**) and composition (phylum; **Table 7**), and genus (**Table 8**) compared to the final blank. Some MFG source-specific effects were observed. For instance, ovine MFGM increased the proportions of *Escherichia/Shigella*, *Oscillobacter*, Fusobacteria, Bacteroides genera, and unclassified Clostridia and decreased the proportions of unclassified *Ruminococcaceae*, *Prevotella*, *Blautia* *Lactobacillus*, and *Clostridium* genera compared to the final blank. PC and caprine MFGM increased the proportions of unclassified Clostridiales, whereas caprine MFGM also increased the proportions of *Oscillobacter* and Fusobacteria compared to the final blank. Higher concentrations of butyric acid were found in the caecal fermentation when incubated with ovine and bovine MFGM compared to other substrates (**Table 9**).

DISCUSSION

In this study, bovine, caprine, and ovine MFGM-enriched fractions were digested with gastric and small intestine enzymes

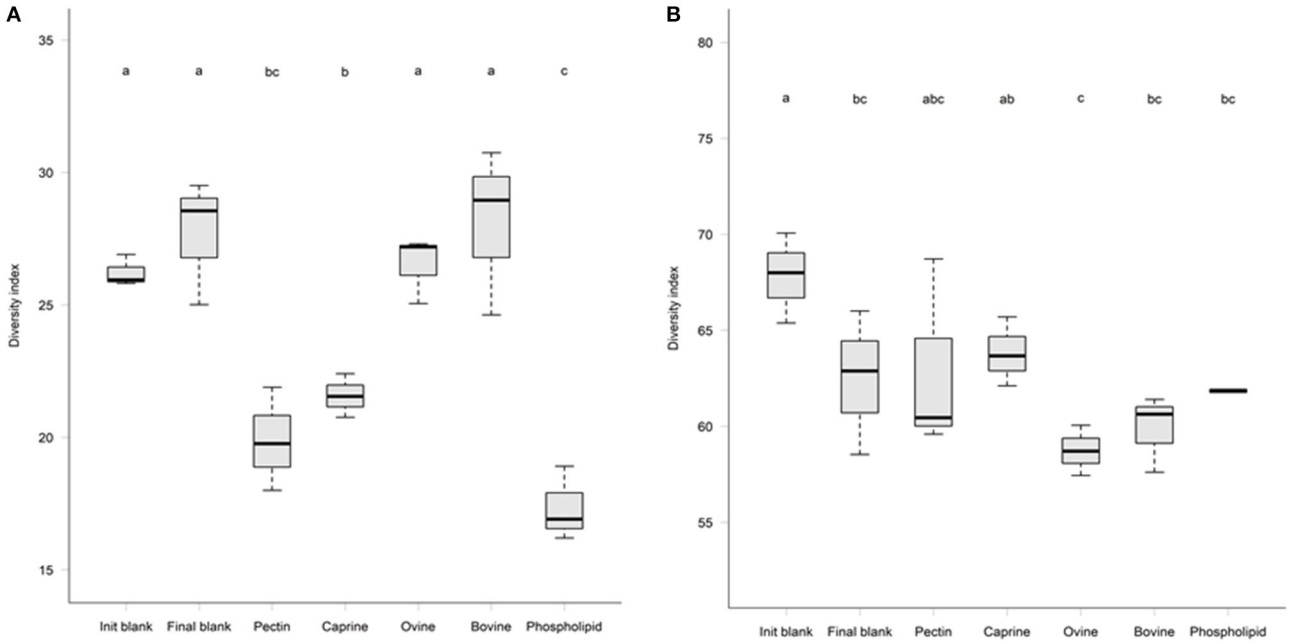

FIGURE 4 | Microbial alpha-diversity after 2 h *in vitro* ileal fermentation **(A)** followed by 12 h of *in vitro* caecal fermentation **(B)** of undigested bovine, caprine, ovine milk fat globule membrane (MFGM), and phospholipid concentrate (PC) in piglets. Pectin was used as a fermentation control. Values are presented as mean ± standard deviation. Means within each without a common letter differ, $P \leq 0.05$.

TABLE 6 | Organic acid production (mmol/kg incubated substrate) after *in vitro* ileal fermentation of digested bovine, caprine, and ovine milk fat globule membrane (MFGM); phospholipid concentrate (PC); and pectin (control substrate).

Organic acid[3]	Substrate[1]					SEM	*P*-value[2]
	PC	Bovine	Caprine	Ovine	Pectin		
Acetic	0.3^{b}	0.0^{b}	0.0^{b}	1.0^{a}	0.0^{b}	0.1	<0.001
Butyric	0.0^{b}	33.0^{a}	0.0^{b}	35.6^{a}	0.0^{b}	0.9	<0.001
Caproic	0.0^{c}	12.0^{a}	0.0^{c}	15.2^{a}	0.0^{c}	0.3	<0.001
Formic	30.7^{a}	25.2^{a}	29.4^{a}	30.7^{a}	5.3^{b}	2.0	<0.001
Lactic	126.4^{a}	121.5^{a}	121.6^{a}	126.8^{a}	23.3^{b}	2.5	<0.001

[1] *Values are means and pooled standard error of the mean (SEMs) for each organic acid, n = 3 for each substrate and organic acid combination.*
[2] *Means within each organic acid without a common letter differ, P ≤ 0.05. Organic acids succinic and propionic acid were not detected in the ileal fermentation.*
[3] *Concentration substrate from values found in the final blank.*

simulating the infant GIT prior to being fermented by ileal and caecal microbiota from piglets fed an infant formula from 11 to 31 days post-natally. As stated in the study's hypothesis, fermentation of MFGM substrates produced organic acids and changed the ileal and caecal microbiota composition in an MFG source-specific manner.

The enriched MFGM fraction obtained in this study contained proteins and polar lipids characteristic of the native MFGM as previously reported for bovine (44), caprine (36), and ovine milk (16). The molecular diversity of lactadherin, for example, was observed in the SDS-PAGE gel with one band identified for caprine and ovine MFGMs and two for bovine MFGM (ranging from 52 to 58 kDa) (45). Higher glycosylation of caprine xanthine oxidase compared to bovine xanthine oxidase may have led to the observation of a xanthine oxidase band in SDS-PAGE gel stained with ProQ Emerald (**Figure 3**, Lane 7 and band a), which has been described elsewhere (36). The composition of commercial PC was different from bovine MFGM with an increased concentration of total proteins and polar lipids, in particular, caseins and phosphatidylserine. Differences in composition between commercial PC and bovine MFG are likely due to variations in enrichment methodology and composition of starting material (46).

This study is the first to report the digestion of a bovine, caprine, and ovine MFGM-enriched fractions using an *in vitro* model with enzymatic concentrations, time of incubation, and pH adapted to simulate conditions found in 5-months old infants (14). Caprine MFGM had a lower recovery after digestion and dialysis (15%) compared to other substrates (35–49%), probably

TABLE 7 | Relative abundances of bacteria at the phylum level in the piglet's caecal microbiota inoculum (initial blank) and after 12 h of incubation alone (final blank) or with bovine, caprine, and ovine MFGM; phospholipid concentrate (PC); and pectin substrates.

Phyla	Initial blank[1]	Final blank[1]	PC[1]	Bovine[1]	Caprine[1]	Ovine[1]	Pectin[1]	*P*-value
Firmicutes	61.6 ± 1.0^{b}	70.1 ± 1.6^{a}	67.0 ± 0.8^{a}	66.8 ± 4.8^{a}	67.7 ± 0.6^{a}	67.0 ± 0.8^{a}	69.2 ± 3.6^{a}	0.04
Proteobacteria	17.3 ± 0.3^{ab}	15.5 ± 0.4^{bc}	16.9 ± 0.2^{abc}	17.2 ± 1.8^{ab}	15.1 ± 1.1^{c}	18 ± 0.5^{a}	16.5 ± 1.4^{abc}	0.06
Bacteroidetes	16.3 ± 0.8^{a}	10.2 ± 0.9^{b}	11.2 ± 1.1^{b}	11.1 ± 2^{b}	11.7 ± 0.1^{b}	10.1 ± 0.7^{b}	10.3 ± 1.6^{b}	0.005
Fusobacteria	2.0 ± 0.4^{abc}	1.2 ± 0.1^{c}	1.9 ± 0^{abc}	2.1 ± 0.7^{ab}	2.4 ± 0.5^{a}	2.2 ± 0.3^{a}	1.3 ± 0.4^{bc}	0.06
Other	0.92 ± 0.04^{abc}	0.8 ± 0.07^{c}	1.07 ± 0.01^{ab}	1 ± 0.15^{ab}	1.1 ± 0.11^{a}	0.96 ± 0.13^{abc}	0.88 ± 0.06^{bc}	0.04
Verrucomicrobia	0.39 ± 0.1^{a}	0.12 ± 0.02^{b}	0.13 ± 0.01^{b}	0.08 ± 0.02^{b}	0.13 ± 0.01^{b}	0.06 ± 0.03^{b}	0.08 ± 0.03^{b}	0
Actinobacteria	0.35 ± 0.02^{bc}	0.59 ± 0.06^{a}	0.30 ± 0.01^{c}	0.42 ± 0.04^{b}	0.36 ± 0.02^{bc}	0.34 ± 0.03^{bc}	0.41 ± 0.10^{bc}	0.003
Spirochaetes	0.35 ± 0.05^{c}	0.38 ± 0.012^{bc}	0.47 ± 0.008^{abc}	0.49 ± 0.09^{b}	0.46 ± 0.07^{abc}	0.54 ± 0.01^{a}	0.37 ± 0.10^{c}	0.031378
Synergistetes	0.31 ± 0.01^{c}	0.45 ± 0.009^{ab}	0.57 ± 0.02^{a}	0.41 ± 0.15^{bc}	0.43 ± 0.03^{bc}	0.47 ± 0.06^{ab}	0.47 ± 0.04^{ab}	0.0396
TM7	0.13 ± 0.02^{b}	0.15 ± 0.02^{b}	0.18 ± 0.04^{ab}	0.14 ± 0.02^{b}	0.22 ± 0.01^{a}	0.16 ± 0.01^{b}	0.13 ± 0.02^{b}	0.0284
Euryarchaeota	0.08 ± 0.02^{b}	0.14 ± 0.03^{a}	0.11 ± 0.01^{ab}	0.07 ± 0.03^{b}	0.15 ± 0.03^{a}	0.07 ± 0.02^{b}	0.08 ± 0.02^{b}	0.0278
Firmicute:Proteobacteria	3.54 ± 0.003^{c}	4.50 ± 0.22^{a}	3.96 ± 0.01^{abc}	3.92 ± 0.67^{abc}	4.49 ± 0.41^{a}	3.72 ± 0.10^{bc}	4.22 ± 0.56^{ab}	0.05

[1] *Values are presented as mean ± standard deviation. Means within each phylum without a common letter differ, P ≤ 0.05.*

TABLE 8 | Relative abundances of significant bacteria classified to the lowest identified taxonomic level in the piglet's caecal microbiota inoculum (initial blank) and after 12 h of incubation alone (final blank) or with bovine, caprine, and ovine MFGM; phospholipid concentrate (PC); and pectin substrates.

Taxa[1]	Initial blank[2]	Final blank[2]	PC[2]	Bovine[2]	Caprine[2]	Ovine[2]	Pectin[2]	*P*-value
Unclassified *Lachnospiraceae* (f)	13.4 ± 1.4^{c}	21.2 ± 1.6^{ab}	20.7 ± 1.5^{ab}	24.2 ± 6.4^{a}	22.8 ± 2.0^{a}	26.0 ± 2.4^{a}	16.9 ± 1.9^{bc}	0.001
Escherichia/Shigella (g)	11.2 ± 0.6^{ab}	9.9 ± 0.4^{cd}	10.9 ± 0.3^{abc}	10.9 ± 0.8^{abc}	9.6 ± 1.0^{d}	11.8 ± 0.3^{a}	10.5 ± 0.7^{bcd}	0.02
Unclassified *Ruminococcaceae* (f)	9.5 ± 0.3^{a}	7.4 ± 0.6^{b}	7.4 ± 0.6^{b}	7.0 ± 0.2^{bc}	7.6 ± 0.1^{b}	6.3 ± 0.3^{c}	7.4 ± 0.4^{b}	0.0006
Unclassified Clostridiales (o)	8.2 ± 0.2^{c}	8.2 ± 0.1^{c}	9.2 ± 0.0^{a}	8.3 ± 0.2^{bc}	8.7 ± 0.1^{ab}	8.1 ± 0.5^{c}	8.1 ± 0.2^{c}	0.005
Prevotella (g)	7.3 ± 0.4^{a}	4.2 ± 0.2^{bc}	3.7 ± 0.6^{cde}	3.5 ± 0.4^{de}	3.9 ± 0.2^{cde}	3.2 ± 0.2^{e}	4.7 ± 0.1^{b}	<0.0001
Megasphaera (g)	4.4 ± 0.4^{a}	1.1 ± 0.2^{b}	1.4 ± 0.1^{b}	1.0 ± 0.1^{b}	1.2 ± 0.1^{b}	1.0 ± 0.0^{b}	1.2 ± 0.1^{b}	<0.0001
Blautia (g)	4.2 ± 0.1^{ab}	4.0 ± 0.3^{ab}	3.9 ± 0.2^{ab}	3.5 ± 0.6^{bc}	3.7 ± 0.5^{b}	2.8 ± 0.3^{c}	3.6 ± 0.7^{bc}	0.02
Unclassified *Enterobacteriaceae* (f)	3.2 ± 0.0^{bc}	3.2 ± 0.1^{bc}	3.3 ± 0.1^{bc}	3.5 ± 0.4^{ab}	2.9 ± 0.1^{c}	3.5 ± 0.2^{ab}	3.4 ± 0.4^{bc}	0.04
Bacteroides (g)	3.2 ± 0.4^{a}	1.6 ± 0.0^{c}	2.5 ± 0.1^{b}	2.9 ± 0.6^{ab}	2.7 ± 0.1^{ab}	2.7 ± 0.2^{ab}	1.7 ± 0.4^{c}	0.0006
Unclassified *Peptostreptococcaceae* (f)	3.1 ± 0.3^{d}	5.7 ± 0.9^{b}	3.1 ± 0.3^{d}	3.7 ± 0.2^{cd}	3.6 ± 0.2^{cd}	3.0 ± 0.2^{d}	8.2 ± 0.7^{a}	<0.0001
Clostridium (g)	2.1 ± 0.2^{cd}	2.9 ± 0.3^{b}	1.4 ± 0.1^{e}	1.7 ± 0.0^{de}	1.8 ± 0.1^{de}	1.4 ± 0^{e}	4.5 ± 0.5^{a}	<0.0001
Unclassified *Prevotellaceae* (f)	2.1 ± 0.4^{a}	1.4 ± 0.2^{bc}	1.4 ± 0.0^{abc}	1.3 ± 0.4^{bc}	1.4 ± 0.1^{ab}	1.0 ± 0.1^{bc}	1.5 ± 0.6^{ab}	0.02
Oscillibacter (g)	2.0 ± 0.2^{d}	3.0 ± 0.2^{c}	3.3 ± 0.1^{bc}	3.2 ± 0.2^{bc}	3.9 ± 0.3^{a}	3.6 ± 0.2^{ab}	3.3 ± 0.3^{bc}	<0.0001
Unclassified Bacteroidetes (p)	1.9 ± 0.0^{a}	1.5 ± 0.3^{ab}	1.8 ± 0.3^{a}	1.8 ± 0.4^{a}	1.9 ± 0.1^{a}	1.7 ± 0.1^{a}	1.2 ± 0.1^{b}	0.009
Fusobacterium (g)	1.8 ± 0.4^{ab}	1.1 ± 0.1^{cd}	1.8 ± 0.0^{abc}	1.9 ± 0.7^{a}	2.2 ± 0.4^{a}	2.0 ± 0.3^{a}	1.2 ± 0.4^{bcd}	0.004
Turicibacter (g)	1.1 ± 0.0^{c}	1.7 ± 0.2^{a}	0.8 ± 0.0^{c}	1.4 ± 0.2^{b}	0.9 ± 0.0^{c}	1.1 ± 0.1^{c}	1.8 ± 0.1^{a}	<0.0001
Lactobacillus (g)	1.0 ± 0.0^{ab}	1.1 ± 0.0^{a}	0.7 ± 0.0^{c}	0.8 ± 0.1^{bc}	0.8 ± 0.1^{c}	0.8 ± 0.1^{c}	1.0 ± 0.1^{ab}	0.003
Coprococcus (g)	0.9 ± 0.1^{b}	1.3 ± 0.0^{a}	1.0 ± 0.0^{b}	1.0 ± 0.1^{b}	1.0 ± 0.1^{b}	0.9 ± 0.1^{b}	1.0 ± 0.1^{b}	0.001
Unclassified Clostridia (c)	0.6 ± 0.0^{c}	1.0 ± 0.0^{b}	1.1 ± 0.0^{ab}	1.0 ± 0.0^{b}	1.0 ± 0.1^{b}	1.2 ± 0.1^{a}	0.8 ± 0.0^{c}	<0.0001
Barnesiella (g)	0.6 ± 0.0^{ab}	0.5 ± 0.0^{abc}	0.6 ± 0.0^{a}	0.5 ± 0.1^{abc}	0.5 ± 0.1^{abc}	0.4 ± 0.0^{c}	0.4 ± 0.0^{c}	0.05

[1] *Taxa identified to the lowest rank; (g) genus, (f) family, (c) class, (o) order, (p) phylum.*

[2] *Values are presented as mean ± standard deviation. Means within each bacterial taxon without a common letter differ, P ≤ 0.05.*

due to a low concentration of both total protein and total lipids in the purified caprine MFGM.

The SDS-PAGE profiles of PC, bovine, caprine, and ovine MFGMs after digestion were similar to the reported data for bovine MFGM digestion using an adult *in vitro* model (10, 15). Different levels of proteolysis after MFGM digestion may be explained by the MFG source-specific profile and concentration of MFGM proteins, especially glycoproteins, in the start material (16, 17, 45). Indeed, a different band pattern in the ProQ-Emerald stained SDS-PAGE gel was observed after the digestion of MFGMs from different sources (bovine, caprine, and ovine). Previous studies using adult *in vitro* digestive conditions showed that MFGM glycoproteins (i.e., butyrophilin, xanthine oxidase, PAS6/7, and mucins) were hydrolyzed to different extents (9, 10). Mucin 1 had the highest resistance to digestion compared to other glycoproteins (i.e., butyrophilin), and a part of this protein

TABLE 9 | Organic acid production (mmol/kg incubated substrate) after *in vitro* ileal and caecal fermentation of undigested bovine, caprine, ovine milk fat globule membrane (MFGM); phospholipid concentrate (PC); and pectin (control substrate).

Organic acid[3]	Substrate[1]					SEM	*P*-value[2]
	PC	Bovine	Caprine	Ovine	Pectin		
Acetic	178.1^{ab}	182.2^{a}	172.4^{ab}	166.4^{b}	151.7^{ab}	4.5	0.007
Butyric	25.2^{b}	41.5^{a}	23.9^{b}	40.1^{a}	20.0^{c}	0.6	<0.0001
Caproic	0.0^{c}	9.6^{b}	0.0^{c}	10.8^{a}	0.0^{c}	0.1	<0.0001
Valeric	9.8^{a}	9.6^{ab}	9.4^{b}	0.0^{c}	9.4^{b}	0.05	<0.0001
Succinic	78.7^{a}	81.7^{a}	81.9^{a}	78.3^{a}	44.1^{b}	1.4	<0.0001

[1] *Values are means and pooled standard error of the means (SEMs) for each organic acid, n = 3 for each substrate and organic acid combination.*

[2] *Means within each organic acid without a common letter differ, P ≤ 0.05. No differences on propionic, formic, and lactic concentrations were observed after incubation with ileal and caecal digesta.*

[3] *Concentration substrates from values found in the final blank.*

was still detected with the original MW after gastric and small intestine digestion (10). Human milk N-glycans were also shown to resist gastrointestinal digestion and were detected in infant stools (47).

Recently, the INFOGEST 2.0 *in vitro* gastrointestinal food digestion method recommended the use of rabbit gastric lipase due to its similar stereospecificity for TAG hydrolysis when compared to human gastric lipase (48). At the time this study was performed, however, rabbit gastric lipase was not commercially available, and the fungal lipases from *Rhizopus orizae* were used. *R. orizae* lipase was shown to have greater lipolysis rate (46 vs. 10%) and be insensitive to FA chain length compared to human gastric enzyme, which released only C8:0 and C10:0 under both gastric and intestinal conditions (49).

To understand the effect of PC and enriched bovine, caprine, and ovine MFGM fractions on ileal and caecal microbiota and production of organic acids, piglet ileal and cacecal inoculum were used to ferment the material remaining after *in vitro* digestion of PC, bovine, caprine, and ovine MFGM preparations. In these *in vitro* assays, PBS was used to homogenize the microbiota instead of nutritive medium used by some authors (50). The PBS is used when a high concentration of feces is used. This high concentration provides the nitrogen and minerals required by the microbiota (51), which must be provided in a nutritive medium when small amount of feces (or cultures) are used. In all our *in vitro* fecal fermentation studies, we have followed the PBS approach to avoid any confounding effect of adding nutrients in a different amount (or ratio) to that in ileal and hindgut (27).

In vitro ileal organic matter fermentation for MFGM substrates showed different fermentability, ranging from 6 to 14%, whereas pectin was higher (28%). Despite the high ileal OM fermentability of pectin, this fermentation produced the lowest concentration of organic acids compared to MFGM and PC. This may indicate fermentation products not measured in this study (e.g., pyruvic acid) could have been produced during pectin fermentation in greater amounts before being converted to other organic acids (e.g., lactic acid) later in the fermentation (52).

In general, Ileal microbiota had substantial proportions of *Turibacter* and *Streptococcus*, which are known lactate producers (53, 54) and may explain the link between ileal lactate and microbiome composition. Bovine and ovine MFGMs produced higher concentrations of acetic, butyric, and caproic acids with limited effects on the ileal microbiota composition. Increase concentration of actinobacteria and bacterioidetes, however, were observed after bovine and ovine MFGM ileal fermentation and may explain the increased concentration of acetic acid observed (55). Butyric acid are mainly produced by members of *Clostridiaceae* family (56), which were also increased after bovine and ovine MFGM fermentation compared to other substrates.

Bovine and ovine substrates reduced the proportions of ileal proteobacteria, which, at elevated levels, have been linked in other studies to dysbiosis (57, 58). Proteobacteria are facultative anaerobes known to consume oxygen, altering the pH and lowering the redox potential, making the intestine suitable for colonization by strict anaerobes. Proteobacteria, in the neonatal small and large intestine, are affected by the type of feeding with a higher frequency of these bacteria in formula-fed infants compared to breast-fed infants [reviewed in (59)].

PC and caprine MFGMs had the largest effect on the ileal microbiota as observed by the reduced alpha diversity, clear separation in the principal coordinate analysis (**Figure S1**), and changes in the taxonomic composition compared to blanks and other substrates. Differences in composition found after digestion in PC (i.e., lower total protein and increased polar lipid concentration, in particular, sphingomyelin and MCFA) and caprine MFGM (lower total fat and polar lipids, in particular very long-chain fatty acids or VLCFA) may have led to the different effects observed in the ileal microbiota. We speculate that components of caprine MFGM and PC may have benefited more dominant members of the ileal microbiota, which consequently may have caused a decrease of rarer microbial taxa, decreasing alpha diversity. Caprine MFGM, for example, increased the ratio of firmicutes:proteobacteria, whereas PC reduced the proportions of unclassified Clostridiales, *Prevotella*, and/or *Lactobacillus* and increased the proportions of *Enterobacteriaceae* in the ileal microbiota compared to the initial and final blanks. These changes are consistent with the microbial profile observed in the ileal microbiota of piglets fed infant formula compared to piglets fed sow milk (60, 61). The

relevance of these findings to human infants, however, still needs to be determined as studies on the ileal microbial in infants are still lacking. In one study, Barret at al. (62) reported that human infant's ileal microbiota is dominated by actinobacteria (94%) up to 40 days of life and then mostly by proteobacteria and firmicutes from 50 to 217 days of life (62).

Fermentation of the organic matter in bovine, caprine, and ovine MFGMs was relatively minor after ileal fermentation (∼3% increased after ileal fermentation) although PC was greater (∼14% after ileal fermentation). Thus, the total ileal–caecal *in vitro* fermentation ranged from 11% (bovine and caprine MFGM substrates) to 19% (PC). Although lower fermentability of organic matter was observed from the caecal microbiota, production of organic acids was observed for all substrates. This may be explained by (1) products of ileal fermentation (i.e., organic acids, glycans) being used by the caecal microbiota as a carbon source, (2) the presence of a larger array of caecal microbial enzymes that are able to degrade resistant non-dietary components (63, 64), and/or (3) longer time of fermentation. Increased concentration of butyric and caproic acids observed after caecal fermentation of bovine and ovine MFGM substrates may originate from ileal fermentation, which was inoculated with caecal microbiota. In contrast, acetate, succinate, and valeric acids, observed for all substrates, are known to be produced by specific microbes [i.e., *Bacteroides* spp., *Prevotella* spp. (65) and *Clostridium* spp (66)], which were present only in the caecal inoculum in the current study.

This study was not designed to demonstrate which specific component (protein, lipid, glycan) of the enriched MFGM from ruminant milks were responsible after *in vitro* gastrointestinal digestion for the effects observed in the ileal and caecal microbiota. However, changes in the proportions of bacterial genus observed in this study are likely due to the combination of fermentable substrates (e.g., glycans) and antimicrobial components (e.g., linolenic acid, C18:3, and polyunsaturated fatty acids or PUFAs) within the enriched MFGM fraction. After digestion, PC, for example, was enriched in sphingomyelin and unsaturated fatty acids (UFAs) compared to the other substrates. Unsaturated fatty acids with 18 carbon long chains—oleic acid (C18:1), linoleic (C18:2), and linolenic acid (C18:3)—have potent antibacterial activities against Gram-positive bacteria (67, 68). Degradation products of sphingomyelin, such as sphingosine, have shown bactericidal activities against *E. coli* and other pathogenic bacteria (69, 70).

In conclusion, the enriched MFGM fractions from bovine, caprine, and ovine milk contain proteins and polar lipids characteristic of the native MFGM. The material of the enriched MFGM fractions remaining after *in vitro* gastric and intestinal digestion, using infant conditions, were mainly fermented *in vitro* by the ileal microbiota, demonstrating the potential importance of ileal fermentation. Fermentation of the MFGM substrates produced organic acids and changed the proportions of the ileal and caecal microbiota in a MFG source-specific manner. Although this study was not designed to identify specific components of the digested MFGMs responsible for the effects on the ileal and caecal microbiota, they are likely to be a combination of fermentable substrates (glycans) and antimicrobial components of the MFGM. More studies are needed to further understand the effects of infant digestion on MFGM components and the *in vivo* effects of MFGM on the ileal and large intestinal microbiota.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in NCBI SRA, NCBI Accession No. PRJNA610043.

ETHICS STATEMENT

The animal study was reviewed and approved by Grasslands Animal Ethics Committee under the New Zealand Animal Welfare Act 1999 (AEC#12997).

AUTHOR CONTRIBUTIONS

CT, CM, NR, and WM designed the study. WY performed the *in vivo* study. CT and CM performed the *in vitro* study. CT supported by CM and WY analyzed the data and wrote the paper, which was reviewed by NR and WM. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2020.00091/full#supplementary-material

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Growth Factor Concentrations in Human Milk Are Associated With Infant Weight and BMI From Birth to 5 Years

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Background: Human milk bioactives may play a role in infant health and development. Although the variability in their concentrations in milk is well-established, the impact of differential milk profiles on infant growth outcomes remains unclear. Thus, the aim of the present study was to investigate whether different concentrations of metabolic hormones are associated with different weight and BMI in infants beyond the first year of life.

Methods: Milk samples at 2.6 (±0.4) months after birth and anthropometric measures at 13 months, 2, 3, and 5 years were collected as part of the Finnish STEPS cohort study from 501 mothers and the respective 507 infants. Leptin, adiponectin, insulin-like growth factor (IGF)-1 and cyclic glycine-proline (cGP) in milk were analyzed. Multiple regression models and a repeated measures mixed model were used to examine associations between milk hormone concentrations and weight and BMI z-scores across time, at each time-point, and weight gain from birth to each follow-up visit. All models were corrected for birth weight, infant sex, duration of exclusive and total breastfeeding, time of introduction of solid foods and maternal pre-pregnancy BMI.

Results: Higher milk IGF-1 was associated with higher weight at 13 months ($p = 0.004$) but lower weight at 3 ($p = 0.011$) and 5 years of age ($p = 0.049$). Higher cGP was associated with lower weight across the 5 years ($p = 0.019$) but with higher BMI at 5 years ($p = 0.021$). Leptin and adiponectin did not display associations with infant growth at this time. Sex interactions were also absent.

Conclusions: Our results suggest that the interplay between human milk-borne IGF-1 and cGP is similar to that reported in other mammals and may have an important role

in defining infant growth trajectories beyond the first year of life. Further research should explore the determinants and origins of these milk-borne compounds and evaluate their effect on infant growth and metabolism.

Keywords: BMI, cGP, human breastmilk bioactives, IGF-1, growth factors, adiponectin, leptin, infant growth

INTRODUCTION

Breastfeeding is the gold standard for infant nutrition. However, the importance of human milk (HM) for infants goes beyond meeting their nutritional needs. It is well-established that HM consumption is associated with lower risk of longer-term morbidities, including obesity (1, 2) and metabolic syndrome (3), which constitute major public health issues worldwide (4). As the concept of nutritional programming during "the first 1,000 days of life" is gaining recognition (5, 6), HM-borne hormones that regulate metabolic function are seen as potential programming factors (7–9). Evidence from animal models suggests that HM leptin (10) influences infant eating behavior and body composition (11) and may underpin the protective effects of breastfeeding against obesity (12). The presence of adipokine receptors in the epithelial cells of the infant gastrointestinal tract (13, 14) together with the knowledge that human neonates are capable of absorbing intact macromolecules during the early neonatal period (15, 16) reinforces the hypothesis that these HM-borne molecules have a functional role in infant metabolism. Additionally, other hormones, including insulin-like growth factor (IGF)-1, together with its binding proteins and regulatory metabolites, including cyclic Glycine-Proline (cGP), also have a potential role in programming infant growth trajectories due to their well-established roles in linear growth, and body composition (17).

As variations in circulating adipokines (18), IGF-1 and cGP (19, 20) have been linked to various health and disease outcomes and significant differences in HM hormone concentrations have been described across mother-infant dyads (21–23), research has focused on associations between hormone concentrations in HM and infant growth outcomes (24–36). However, while some studies suggest that individual hormonal signatures in HM are associated with different infant growth trajectories, the findings of research in this area remains conflicting (37). Yet, the pattern of early postnatal growth beyond the first year of life is a known predictor of later metabolic health (38) and previous studies have shown that the composition of milk received by the infant plays an important role in determining growth trajectories (39).

In a recent study we reported on the association between HM concentrations of adiponectin, leptin, IGF-1 and cGP with maternal-infant characteristics in the STEPS cohort (40). We identified significant differences in the concentrations of HM-borne total protein, IGF-1, leptin and adiponectin across mother-infant dyads, particularly in association with maternal/perinatal/infant characteristics including pre-pregnancy BMI, maternal education, birth mode, gestational diabetes (GDM), and infant sex. Following our initial observations, in the present study we hypothesized that differences in HM composition would correlate with different weight and BMI z-scores in the infants from birth to 5 years of age.

MATERIALS AND METHODS

Study Design and Population Characteristics

This study utilizes HM samples obtained from 501 mothers and 507 children participating in the Finnish longitudinal cohort, Steps to healthy development of Children (the STEPS Study) between 2008 and 2010, as described previously (40). HM samples from consenting mothers were collected 2.6 $\pm$ 0.4 months after birth by manual expression in the morning, from a single breast, as detailed previously (41). Study visit retention decreased from birth (N = 507) to 5 years (N = 391) but remained high at almost 80%. Maternal pre-pregnancy BMI was calculated from self-reported height and weight and clustered into the following categories: underweight < 18.5 kg/m^2, normal weight 18.5–24.9 kg/m^2, overweight 25–29.9 kg/m^2, obese >29.9 kg/m^2. Information regarding infant sex, birth weight and length were obtained from the Longitudinal Census Files. Information on feeding practices was collected through follow-up diaries completed by mothers (42). Exclusive breastfeeding was defined as the infant not receiving anything other than HM, with the exception of water, supplements or medicines. Total breastfeeding was defined as the infant receiving HM and any other liquid or food. Infant anthropometric measures (weight, length/height) were taken during follow-up visits at 13 months, 2, 3, and 5 years of age as previously detailed (42). These measurements were used to calculate the BMI and respective z-scores using references specific to the Finnish population (43). Weight gain was calculated as difference between each clinical visit and birth weight. The study protocol was approved by the Ethics Committee of the Hospital District of Southwest Finland in February 2007 and March 2015. Written informed consent was obtained from all participants.

HM Analysis

HM analysis was undertaken in 2018 as described previously (40). In brief, leptin, adiponectin and IGF-1 in HM were analyzed using commercially available ELISA kits (human sensitive leptin ELISA, human adiponectin ELISA, human IGF-1 ELISA, Mediagnost, Germany), while cGP was analyzed via liquid chromatography tandem mass spectrometry (LC-MS). HM total protein was quantified by infrared spectrometry using the Direct Detect® technology (Merck, Germany) in order to normalize hormone concentrations. The intra- and inter-assay coefficients of variation, respectively, for the ELISAs (QCs supplied) were adiponectin (5, 6%), IGF-1 (3, 9%), and leptin (4, 8%).

TABLE 1 | Characteristics for the study population at each clinical visit.

Population characteristics	Birth	13 months	2 years	3 years	5 years
Anthropometry, Mean (SD)					
N (%)	507 (100)	504 (99)	448 (88)	405 (80)	391 (77)
Missing (%)	0	3 (1)	59 (12)	102 (20)	116 (23)
Weight (kg)	3.490 (0.506)	10.352 (1.108)	12.941 (1.351)	15.260 (1.613)	19.987 (2.521)
Weight (z-score)	0.18 (0.889)	−0.19 (1.197)	0.08 (0.964)	0.09 (0.938)	0.22 (0.901)
BMI (kg/m^2)	NA	NA	16.5 (1.2)	16.2 (1.1)	16.0 (1.3)
BMI (z-score)	NA	NA	0.04 (1.048)	0.21 (0.958)	0.08 (0.897)
Weight gain (kg)	NA	6.86 (1.05)	9.44 (1.31)	11.76 (1.52)	16.49 (2.43)
Infant sex, *N* (%)					
Male	273 (54)	270 (54)	245 (55)	219 (54)	212 (54)
Female	234 (46)	234 (46)	203 (45)	186 (46)	179 (46)
Maternal pre-pregnancy BMI, *N* (%)					
Underweight	20 (4)	19 (4)	20 (4)	19 (5)	16 (4)
Normal weight	343 (68)	343 (68)	302 (67)	281 (69)	277 (71)
Overweight	91 (18)	91 (18)	85 (19)	70 (17)	63 (16)
Obese	51 (10)	51 (10)	41 (9)	35 (9)	35 (9)

Statistical Analyses

For the purpose of this study we only used weight and BMI z-scores and weight gain as outcome variables, since these are the primary predictors for childhood obesity (44). The power provided by the smallest sample size ($n = 391$) amongst the follow-up visits was above 90% for multiple linear regression analyses at 5% significance level for the detection of small effect sizes (G*Power 3.1.9.2). Similar to our previous study, HM concentrations of leptin adiponectin, IGF-1 and cGP were corrected for total protein concentration in each sample (mg/ml) and reported as ng/mg of protein per ml (ng/mg). Outcome variables were assessed for normality with the Shapiro Wilk test. Since the distribution of weight gain was not normal, all weight gain variables were log10 transformed. Associations between adipokine and growth factor concentrations in HM and weight and BMI z-scores from 13 months to 5 years were analyzed using multiple linear regression models (to assess associations between HM composition and individual infant outcomes at single time-points) and repeated measures mixed models (to assess associations between HM composition and individual infant outcomes across time-points from 13 months to 5 years of age). Associations between hormone concentrations in HM and weight gain from birth to each clinical visit (13 months, 2, 3, and 5 years) was also analyzed through multiple linear regression models. Interactions of HM composition with infant sex were examined in each model. Maternal BMI class before pregnancy, birth weight, total duration of breastfeeding as well as the time of introduction of solid foods were used as correcting factors (45, 46) (fixed factors) in each regression model (both multiple linear regression and repeated measures), as they were significantly associated with infant growth outcomes which was the dependent variable in our cohort (weight z-scores, BMI z-scores and weight gain) during simple regression analysis. Gestational age and birth-mode were also considered as co-factors. However, since both variables strongly correlated with birth weight, we only used the latter (47). No random effects were evaluated in this study, but the use of repeated measures mixed models was adopted in order to appropriately deal with the missing data (e.g., any dropouts between clinical visits). All statistical analyses were performed using IBM SPSS (version 25).

RESULTS

Population Characteristics

Of the 507 infants whose mothers had consented for HM hormone and growth factor analysis, 391 (77%) were followed up until 5 years of age.

The overall characteristics of the study population are reported in **Table 1**. Although the number of infants decreased from one visit to the other, the proportion in infant sex, and maternal pre-pregnancy BMI remained consistent throughout the study. As shown by the mean z-scores for weight and BMI, our study population did not differ from the general Finnish population.

HM Adipokine and Growth Factor Composition and Infant Growth

As shown in **Table 2**, higher concentrations of HM IGF-1 were associated with higher weight at 13 months and lower weight at 3 and 5 years, while higher milk-borne cGP was associated with lower weight at 13 months and overall with lower weight z-scores across the 5 years, as indicated by the results of the repeated measures regression (**Table 2**). Higher IGF-1 was also associated with lower weight gain from birth to 2 years (**Table 3**) and higher cGP was associated with higher BMI at 5 years of age. No significant associations were found between HM leptin and adiponectin and infant growth and there were no sex-specific interactions (data not shown).

TABLE 2 | Associations between weight z-scores at each follow-up visit and over time and HM composition.

	Weight z-scores									
	Weight 13 months		Weight 2 years		Weight 3 years		Weight 5 years		Weight z-score across time	
HM composition	**B (95% CI)**	***p***	**B (95% CI)**	***p***	**B (95% CI)**	***p***	**B (95% CI)**	***P***	**B (95% CI)**	***p***
Leptin (ng/mg)	0.019 (−0.058, 0.096)	0.628	−0.019 (−2.018, 1.980)	0.985	0.088 (−2.172, 2.348)	0.939	0.227 (−1.917, 2.372)	0.835	0.020 (−0.062, 0.103)	0.623
Adiponectin (ng/mg)	0 (−0.017, 0.018)	0.955	0.236 (−0.169, 0.640)	0.245	0.304 (−0.124, 0.732)	0.164	0.242 (−0.171, 0.655)	0.25	0.108 (−0.178, 0.395)	0.745
IGF-1 (ng/mg)	**0.033 (0.011, 0.056)**	**0.004**	−0.367 (−1.020, 0.286)	0.27	**−0.961 (−1.705, 0.218)**	**0.011**	**−0.742 (−1.471, −0.014)**	**0.046**	0.026 (−0.013, 0.065)	0.187
cGP (ng/mg)	**−0.024 (−0.042, −0.006)**	**0.009**	−0.324 (−0.790, 0.143)	0.174	0.075 (−0.327, 0.134)	0.714	0.304 (0.784, 1.538)	0.215	**−0.023 (−0.042, −0.004)**	**0.019**
IGF-1/cGP ratio	**0.006 (0.001, 0.012)**	**0.018**	0.035 (−0.128, 0.199)	0.674	−0.109 (−0.228, 0.011)	0.075	−0.097 (−0.228, 0.034)	0.146	0.005 (−0.005, 0.015)	0.292
Protein (mg/ml)	$3.817 * 10^{-05}$ (−0.002, 0.002)	0.962	−0.017 (−0.059, 0.024)	0.414	−0.016 (−0.058, 0.541)	0.462	−0.022 (−0.060, 0.017)	0.265	0 (−0.002, 0.002)	0.883

Values represent parameter estimates obtained by each individual fitted regression model with the following cofactors: maternal pre-pregnancy BMI, birth weight, infant sex, total breastfeeding duration and introduction of solid foods. IGF-1, Insulin-like growth factor-1; cGP, cyclic Glycine Proline; B, unstandardized beta coefficients. BMI and weight z-scores were calculated using reference values specific to the Finnish population (43). Weight gain was calculated as difference between each clinical visit and birth weight. Bold values indicate significant p-values.

DISCUSSION

The present study shows that HM bioactive concentrations 3 months after birth were significantly associated with infant growth, even when adjusted for important confounding factors including birth weight, maternal pre-pregnancy BMI, total duration of breastfeeding and timing of introduction of solid foods. Our results suggest that milk-borne IGF-1 and cGP are related to weight and z-scores up to 5 years of age and weight gain up to 2 years of age.

While IGF-1 is the main growth factor during infancy (48), results on the relationship between different concentrations of IGF-1 in HM and infant growth outcomes are conflicting (49, 50). In the current cohort, concentrations of IGF-1 in HM collected 3 months after birth were associated with higher weight z-scores at 13 months and associated with lower weight z-scores at 3 and 5 years of age. Previous work by Ong et al. reported that circulating concentrations of IGF-1 at 5 years of age were inversely correlated to birth weight and positively correlated to current weight and weight gain from birth to 2 years of age, concluding that circulating IGF-1 in childhood is linked to growth rates in infancy (51). In the present study we found that HM IGF-1 concentrations were significantly associated with the same outcomes (i.e., weight, weight gain) but in the opposite direction compared to serum IGF-1. This suggests that HM IGF-1 could be involved in production pathways for endogenous IGF-1.

In this context, previous studies in both experimental animal models and clinical settings have reported that circulating IGF-1 during infancy is strongly regulated by nutrition (52, 53). In particular, breastfed infants have lower circulating IGF-1 and lower weight gain compared to formula fed infants (53). The only study to our knowledge that has examined IGF-1 concentrations in infant formula reported undetectable amounts of bovine IGF-1 [identical to human IGF-1 (54)] in the milk used in the formula production (55). While we do not know if bovine IGF-1 would be active in human infants or not (56), the presence of IGF-1 in HM as opposed to its absence/inactivity in infant formula might contribute to differences in endogenous IGF-1 regulation between breastfed and formula-fed infants.

While the impact of dietary IGF-1 on endogenous IGF-1 synthesis is not known, a previous study in a murine IGF-1R knock out model suggested an effect of IGF-1 signaling in adipocytes on systemic concentrations of IGF-1 (57). Such effects on systemic concentrations of IGF-1 was independent of alterations in growth hormone (GH) secretion and resulted in changes in somatic growth. Interestingly, decreased IGF-1 signaling resulted in higher weight gain and adipocyte hypertrophy. The study also found that increased IGF-1 signaling exerted a negative feedback on circulating IGF-1 (57). In light of this, we speculate that dietary and HM IGF-1 during early life might play a role in adipose tissue IGF-1 signaling, so that higher HM concentrations of IGF-1 may downregulate circulating IGF-1 and reduce infant weight gain and weight z-scores in the long term. Although in the present study we did not have the means to examine circulating concentrations of IGF-1 in the infants, it is possible that HM IGF-1 may explain why breastfed infants display lower circulating

TABLE 3 | Associations between weight gain and BMI z-scores and HM composition.

	Δ Weight from birth [log10(Kg)]							
	Weight gain from birth to 13 months		**Weight gain from birth to 2 years**		**Weight gain from birth to 3 years**		**Weight gain from birth to 5 years**	
HM composition	**B (95% CI)**	***p***	**B (95% CI)**	***p***	**B (95% CI)**	***p***	**B (95% CI)**	***p***
Leptin (ng/mg)	−0.008 (−0.142, 0.125)	0.905	0 (−0.146, 0.145)	0.995	0.012 (−0.131, 0.155)	0.872	0.073 (−0.087, 0.232)	0.371
Adiponectin (ng/mg)	0.016 (−0.011, 0.043)	0.256	0.018 (−0.009, 0.045)	0.193	0.014 (−0.012, 0.040)	0.281	0.002 (−0.027, 0.031)	0.882
IGF-1 (ng/mg)	−0.023 (−0.068, 0.021)	0.305	**−0.060 (−0.107, −0.014)**	**0.011**	−0.047 (−0.094, 0)	0.051	−0.050 (−0.106, 0.006)	0.083
cGP (ng/mg)	−0.022 (−0.055, 0.010)	0.177	0.005 (−0.021, 0.030)	0.73	0.019 (−0.012, 0.050)	0.228	0.009 (−0.028, 0.047)	0.627
IGF-1:cGP ratio	0.002 (−0.009, 0.013)	0.667	−0.007 (−0.014, 0.001)	0.075	−0.006 (−0.015, 0.002)	0.148	−0.009 (−0.019, 0.002)	0.102
Protein (mg/ml)	−0.001 (−0.004, 0.002)	0.428	−0.001 (−0.004, 0.002)	0.435	−0.001 (−0.004, 0.001)	0.243	−0.003 (−0.006, 0)	0.086
	BMI z-scores							
	BMI 2 years		**BMI 3 years**		**BMI 5 years**		**BMI across time**	
HM composition	**B (95% CI)**	***p***	**B (95% CI)**	***p***	**B (95% CI)**	***p***	**B (95% CI)**	***p***
Leptin (ng/mg)	0.522 (−1.898, 2.942)	0.672	−0.643 (−2.675, 1.390)	0.535	0.067 (−2.0.40, 2.175)	0.95	0.059 (−1.477, 1.596)	0.939
Adiponectin (ng/mg)	0.039 (−0.435, 0.513)	0.871	0.348 (−0.053, 0.749)	0.089	0.089 (−0.315, 0.492)	0.666	0.104 (−0.235, 0.442)	0.548
IGF-1 (ng/mg)	−0.818 (−1.755, 0.119)	0.087	−0.542 (−1.336, 0.252)	0.181	−0.480 (−1.270, 0.309)	0.233	−0.615 (−1.301, 0.070)	0.078
cGP (ng/mg)	−0.213 (−0.813, 0.387)	0.486	0.276 (−0.144, 0.696)	0.197	**0.507 (0.076, 0.938)**	**0.021**	0.149 (−0.202, 0.500)	0.405
IGF-1:cGP ratio	0.020 (−0.191, 0.232)	0.851	**−0.212 (−0.312, −0.113)**	***p* < 0.001**	**−0.235 (−0.350, −0.120)**	***p* < 0.001**	−0.130 (−0.300, 0.040)	0.133
Protein (mg/ml)	−0.015 (−0.067, 0.038)	0.583	−0.010 (−0.056, 0.035)	0.656	−0.017 (−0.061, 0.026)	0.435	−0.013 (−0.047, 0.020)	0.436

Values represent parameter estimates obtained by each individual fitted regression model with the following cofactors: maternal pre-pregnancy BMI, birthweight, infant sex, total breastfeeding duration and introduction of solid foods. IGF-1, Insulin-like growth factor-1; cGP, cyclic Glycine Proline; B, unstandardized beta coefficients. BMI and weight z-scores were calculated using reference values specific to the Finnish population (43). Weight gain was calculated as difference between each clinical visit and birth weight. Bold values indicate significant p-values.

IGF-1 concentrations as compared to formula-fed infants. Considering that weight gain during infancy is a predictor of adiposity later in life (38) further studies investigating the role of HM IGF-1 as a mediator of weight gain during infancy are required.

While we did not measure IGF-1 binding proteins in our cohort, we analyzed cGP, a metabolite of IGF-1. cGP has been reported to play a regulatory function in relation to IGF-1 activity by maintaining IGF-1 homeostasis through competing against IGFBP-3 binding sites on IGF-1 (58). In our study, the analysis of the associations between weight and BMI z-scores and cGP concentrations in HM highlights a reversed action of cGP compared to that observed for IGF-1. This, together with the fact that both cGP and IGF-1 concentrations in HM were significantly associated with infant growth, further suggests that cGP may be modulating IGF-1 bioactivity in humans, as reported previously, with the ratio between the two indicating IGF-1 bioactivity (58). In this context, a higher ratio between IGF-1 and cGP (IGF-1/cGP), was associated with BMI at 3 and 5 years of age. Lastly, higher HM cGP concentrations were associated with lower weight z-scores trajectories over time suggesting that cGP may exert regulatory effects on IGF-1 activity in both directions (i.e., cGP may enhance the activity of IGF-1 when at low concentrations and reducing activity when IGF-1 is in excess).

Neither leptin nor adiponectin displayed significant associations with infant growth outcomes in the long term, despite some studies suggesting that these compounds may also have a role in infant development and growth (28, 59, 60). Overall, the existing literature in this context is conflicting [see (37) for review] and disparities in the findings are, in part, likely due to different approaches as regards sample collection and storage, laboratory approaches used and statistical analysis of the data, as infant growth is influenced by a complex array of factors. While some of these, including basic characteristics, that can be identified and controlled for, other factors, including potential synergism/antagonism between our hormones of interest and other bioactive factors in HM (37), are difficult to control for. The lack of associations in the present cohort between HM adipokine concentrations and infant BMI and weight does not preclude associations with infant body composition *per se*, as leptin in particular has been linked to eating behavior and fat mass (11).

The main strength of the present study was the large sample size and the prolonged follow-up data that allowed us to investigate associations between HM composition around 3 months postpartum and infant growth trajectories up to 5 years of age. Unfortunately, due to the lack of infant blood samples, we could not investigate the relationship between maternal HM bioactive concentrations and the respective circulating concentrations in the infant. Furthermore, while we were able to detect the concentrations in the samples given, the actual amount of bioactive IGF-1 received by the infant (i.e., not bound to binding proteins that limit its bioavailability) remains unknown. Certainly, the collection of maternal and infant blood samples as well as that of HM at more than one time-point would have aided in the formulation of hypothesis around possible mechanisms underlying our observations. While only one HM sample per mother was available, this was representative of mature milk, which is fairly stable across lactation (61), thus ideally representative of the entire lactation period from the first month to weaning. Of note, despite HM collection occurring on average 3 months after birth, we did not find any association between hormone concentration and exact time of collection, indicating that hormone concentrations were stable across slightly different collection times. The interpretation of the current findings are also limited by a number of factors (e.g., infant diet and physical activity) that were not available to us and that will impact on growth trajectories up to 5 years. Evaluating the eating behavior and body composition of the infant would also been valuable in relation to HM leptin.

Overall, although the variation in BMI and weight z-scores in the present population was generally small with values proximal to zero, IGF-1, and cGP displayed significant associations with infant growth outcomes. This, together with evidence arising from other studies in the same cohort (62), suggests a role for HM-borne compounds in contributing to postnatal growth trajectories. As the role of IGF-1 in association with adiposity in infancy remains poorly understood and current data remain conflicting, more comprehensive studies are required to investigate the mechanistic role of IGF-1 and cGP in HM and early development.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committee of the Hospital District of Southwest Finland in February 2007 and March 2015. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LG designed the research questions, carried out laboratory and statistical analysis, interpreted the results, and drafted the manuscript. HL designed sample and data collection, assisted with statistical analysis, and contributed to the manuscript development. AM and CR helped design the research question and perform assay validations, and together with DC-S and SR edited the final manuscript. MV and SP supervised the entire process from laboratory analysis to submission of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Human Milk Glucocorticoid Levels Are Associated With Infant Adiposity and Head Circumference Over the First Year of Life

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Human milk (HM) is a complex and dynamic biological fluid, which contains appreciable concentrations of the glucocorticoids, cortisol and cortisone. Experimental studies in non-human primates suggest the HM glucocorticoids' impact on infant growth and body composition. In this current study, analysis is made of the relationships between HM glucocorticoid concentrations and the infant growth and development over the first year of life. HM was collected by lactating healthy women ($n = 18$), using a standardized protocol, at 2, 5, 9, and 12 months after childbirth. Cortisol and cortisone concentrations in the HM were measured using liquid chromatography mass spectrometry. Infant weight, length and head circumference were measured by standard protocols and percentage fat mass (% FM) determined by whole body bioimpedance. Cortisol and cortisone concentrations were unaltered over the analyzed lactation period (2–12 months), and were altered by infant sex. Although, HM cortisol was positively associated with infant percentage fat mass (% FM) ($p = 0.008$) and cortisone positively associated with infant head circumference ($p = 0.01$). For the first 12 months of life, the concentration of HM glucocorticoids levels was positively associated with infant adiposity (%FM) and head circumference. This preliminary evidence provides insight to a possible relationship between ingested HM glucocorticoids and infant body composition. Further studies are required to determine the mechanisms regulating HM glucocorticoids.

Keywords: cortisol, cortisone, lactation, mass spectrometry, fat mass, head circumference

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INTRODUCTION

Nutrition during the first 1000 days of life, a period from conception to the child's second year, has a major impact on the infant's growth and development (1). It is during this critical period of life where subtle changes in growth and developmental trajectories can have substantial impact on the health of that individual later in life, including obesity and non-communicable disease risks through childhood and into adulthood (2, 3). Numerous studies demonstrate that the infant intake of mothers' milk (human milk: HM) and establishment of breastfeeding for longer periods confer benefits for both the mother and infant, from nourishment, cognitive benefits, immune protection and reduces the risk of childhood obesity (4–8).

Human milk (HM) is complex and dynamic, not only containing nutritive factors (9), but also being recognized as a rich source of hormones, which have been demonstrated to impact directly or indirectly on infant body functions (10). The presence of hormones, such as leptin, insulin, ghrelin, adiponectin, and insulin like growth factor-1 (IGF-1) is of great importance, due to their involvement in the key aspects of appetite and metabolic regulation (10–13). Thus, amoungst infants where HM is the predominant source of nourishment for the first year of life, subtle variation in HM composition, may modify early growth, and development.

One major class of hormones present in HM are the glucocorticoids, cortisol, and cortisone. All are involved in the regulation of metabolic homeostasis and inflammation (14). However, far less is known of the potential biological functions of HM derived glucocorticoids in the developing infant. It has been demonstrated that the glucocorticoids are predominantly transferred from the maternal circulation into HM. Unlike plasma, the concentration of cortisone in HM is higher than cortisol and also greater than the levels measured in the maternal plasma circulation (15–17). Furthermore, differences exist in salivary cortisol concentrations between breastfed infants and formula feed infants (18) and plasma cortisol strongly correlates with HM cortisol over the first year of life (15). Whilst the role of HM cortisol has not been fully elucidated, HM cortisol correlates with infant mood and aspects of behavioral development, including sex specific temperament issues (19, 20). Hahn et al. (21) demonstrated that higher HM cortisol was associated with lower body mass index percentile (BMIP) at 2 years of age, suggesting that HM cortisol is protective against later life excess adiposity. However, it is difficult to examine closely the impacts of HM glucocorticoid composition and growth and body composition in toddlers, given the increased role exerted by increasing solid food and supplemental milk, including varying formula based beverages on the childs development (22). Therefore, studies are still required in younger children.

Much is known about the nutritional composition of HM and its dynamicity, to meet the changing demands of infants at every stage of lactation (9, 23, 24). Studies have provided considerable details of the changing HM macronutrients over the first 12 months of lactation (25). There remains little data on the variation and regulation of HM glucocorticoids throughout lactation. To the best of our knowledge, there has been only one human study (26) that has analyzed the concentrations of cortisol over an extended period of 12 months of lactation. In this study, HM samples were analyzed to determine the changes in HM cortisol measured by radioimmunoassay and corticosteroid binding globulin (CBG) assay at different stages of lactation, largely between late pregnancy and after the cessation of breastfeeding, reporting lower concentrations during the established lactation (26). However, this study did not quantify cortisone, the predominant glucocorticoid in HM, nor did it examine the relationship between HM glucocorticoids and infant growth. Therefore, in the current study, we aimed to measure the concentration of HM cortisol and cortisone using liquid chromatography mass spectrometry (LC-MS/MS), and investigate their relationships with the development of breastfed infant body composition and growth over the first 12 months of life.

METHODS

Study Design and Subjects

Between 2013 and 2015, breastfed infants ($n = 18$) of predominantly Caucasian and mothers of higher social-economic status were recruited from the community, primarily via the Australian Breastfeeding Association, Perth, Western Australia. Inclusion criteria were: healthy singletons, gestational age $\geq$ 37 weeks, exclusively breastfed at 2 and 5 months (27), and maternal intention to breastfeed until 12 months, without the introduction of formula. Exclusion criteria were: infant factors that could potentially influence growth and development of BC, maternal smoking and low milk supply. All mothers provided written informed consent to participate in the study, which was approved by The University of Western Australia Human Research Ethics Committee (RA/1/4253, RA/4/1/2639) and registered with the Australian New Zealand Clinical Trials Registry (ACTRN12616000368437).

Maternal and infant anthropometric measurements were made at the time of sample collection. Participants visited the research laboratory at King Edward Memorial Hospital for Women (Subiaco, Perth, WA) for up to 4 monitored breastfeeding sessions between March 2013 and September 2015. At each study session, the infants were weighed pre-feed, and then the mother breastfed her infant. Infant bioelectrical spectroscopy (BIS) measurements were taken pre-feed, unless impractical, then they were taken post-feed (28). Anthropometric measurements were taken post-feed. Clothing was removed for the measurements except for a dry diaper and a singlet.

Anthropometric Measurements

Infant's weight was determined before breastfeeding using Medela Electronic Baby Weigh Scales (±2.0 g; Medela Inc., McHenry, IL, USA), whereas maternal weight was measured using Seca electronic scales (±0.1 kg; Seca, Chino, CA, USA). Maternal and infant BMI was calculated as Bodyweight (kg)/(Height (m))^2. Infant crown-heel length was measured once to the nearest 0.1 cm using non-stretch tape and a headpiece and a foot piece, both applied perpendicularly to the hard surface. Infant head circumference was measured with a non-stretch tape to the nearest 0.1 cm. Maternal height measured against a calibrated marked wall (accuracy∼0.1 cm).

Bioimpedance Spectroscopy Measurements

Whole body bioimpedance (wrist to ankle) of infants and mothers was measured using a bioelectrical impedance analyzer (ImpediMed SFB7, Brisbane, Queensland, Australia). Mothers were measured in a supine position on a non-conductive surface. A series of ten consecutive measurements of percentage fat mass (% FM) were taken within 1–2 min and averaged for data analysis. Within the participant coefficient of variation (CV) for maternal % FM was 0.21% (29). Infants' whole body bioimpedance was measured by applying an adult protocol as used previously with data

analyzed using settings customized for infants (30, 31). Values of resistance (ohm) at a frequency of 50 kHz (R_{50}) were determined from the curve of best fit, averaged for analysis purposes and used in BIS age-matched equations for fat-free mass. BIS-based prediction equations for infant BC (31–33) were sourced from the literature, evaluated compared with the reference data (30) and selected according to the following criteria: the absence of significant difference from the reference distribution, closest age match, predominantly Caucasian population. Within participant CV for infant R_{50} was 1.5% (28).

Milk Collection

All sample sets with the exception of one were collected between 9:30 and 10:30 am at the time of measurements at King Edward Memorial Hospital for Women (Perth, Western Australia) at 2 and/or 5, 9, and 12 months postpartum. Small (1–2 mL) pre- and post-feed milk samples were collected into polypropylene 5 mL polypropylene vials (Disposable Products, Adelaide, Australia) and frozen at −20°C. Samples were shipped on dry ice to The University of Auckland, Auckland, New Zealand for glucocorticoid analysis, and were subsequently kept frozen at −80°C until analysis.

Milk samples were divided into four intervals and were classified as T2 (samples collected between 1.9 and 2.3 months) (13 milk samples), T5 (samples collected between 4.8 and 5.5 months) (18 milk samples), T9 (samples collected between 8.8 and 9.8 months) (18 milk samples) and T12 (samples collected between 11.6 and 12.7 months) (13 milk samples). These time periods were used in the subsequent analysis as four major time points. Milk samples were analyzed for all participants on a minimum of 3 timepoints.

HUMAN MILK GLUCACORTICOID ANALYSIS

Sample Preparation

HM steroids were measured by liquid chromatography mass spectrometry (LC-MS), as described previously (17). The internal standard consisted of 12 ng/ml cortisol d4, 60 mg/ml corticosterone d8, prepared in water. All milk samples were warmed to 37°C and vortexed for 10 s before 100 µl of the sample was added to a glass tube containing 100 µL of the internal standard solution. Steroids were then extracted using 1 ml ethyl acetate (Merck, Germany); the top organic layer was removed into a separate tube and then vacuum dried (Savant, SC250EX, Thermo Scientific, USA) for ∼1 h. The dried residues were reconstituted with 80 µl of 50% methanol (Merck, Germany)/water and transferred to HPLC injector vials. All samples were run in duplicate, and average values are reported.

Liquid Chromatography Tandem Mass Spectroscopy

The HPLC tandem mass spectrometer (MS) used an Accela MS pump and auto sampler followed by an Ion Max APCI source on a Thermo Scientific Quantum Ultra AM triple quadrupole mass spectrometer, all controlled by Finnigan Xcaliber software (Thermo Electron Corporation, San Jose, CA.). The mobile phase was a methanol-water gradient starting at 60:40(v/v) (peaking at 80:20 before returning back to 60:40) at 300 µl/min. The chromatography was performed using a Phenomenex Luna C18 (2)-HST column (100 × 3 mm, 2.5 µm particle size) at 40°C. The instrument was set up in selective reaction monitoring (SRM) mode with the following mass transitions: m/z 363.2 → 121.09 for cortisol, 361.1 → 163.04 for cortisone, 367.1 → 121.04 for cortisol d4 and 355.2 → 125.10 for corticosterone d8. Dissociation voltage was 24V, and the collision gas (Argon) was set at 1.2 m Torr for all steroids. Steroid concentrations were calculated from a standard curve generated for each steroid relative to its internal standard (cortisol d4 for cortisol and corticosterone d8 for cortisone).

Statistical Analysis

Results are expressed as mean ± SD unless mentioned otherwise. Normality was checked and normal distribution was observed. ANOVA was used to compare the differences in cortisol and cortisone concentration between different stages of lactation. Spearman correlation was run to assess the relationship between HM glucocorticoids and maternal and infant characteristics. Linear mixed models were employed to investigate associations between HM glucocorticoids concentration and both maternal and infant characteristics. Linear regression was performed at four timepoints to investigate associations with HM glucocorticoids. A $p \leq 0.05$ was considered significant. There was no imputation carried out for missing values. Means and standard deviations were used for reporting. Statistical analysis was carried out using SPSS software (SPSS version 23.0 for

TABLE 1 | Maternal (M) and infant (Inf.) characteristics of longitudinal study measuring the concentration of glucocorticoids in HM samples at T2, T5, T9, and T12 months postpartum.

	Mean ± SD			
Mothers ($n = 18$)				
Age (years)	33.88 ± 4.88			
Lactation stage (months)	T2.0	T 5.0	T 9.0	T 12.0
M weight (kg)	73.06 ± 17.35	62.79 ± 16.34	68.02 ± 18.66	65.83 ± 20.71
M %FM (BIS)	35.04 ± 5.27	32.21 ± 7.07	31.68 ± 7.24	28.97 ± 7.47
Infant sex	Female ($n = 8$)		Male ($n = 10$)	
Inf. Length (cm)	57.64 ± 1.95	64.76 ± 2.35	70.25 ± 2.05	74.15 ± 2.43
Inf. Weight (kg)	5.59 ± 0.88	7.46 ± 0.98	8.76 ± 0.95	9.66 ± 0.79
Inf. BMI	16.30 ± 1.38	17.74 ± 1.70	17.59 ± 1.58	17.30 ± 1.116
Inf. Head circum. (cm)	39.62 ± 1.35	42.91 ± 1.72	45.45 ± 1.68	46.32 ± 1.42
Inf. %FM (BIS)	21.74 ± 2.17	28.24 ± 3.26	25.13 ± 4.65	24.26 ± 3.35

BIS, bioelectrical impedance spectroscopy; BMI, body mass index; %FM, percentage fat mass; T2 (1.9–2.3 months), T5 (4.8–5.5 months), T9 (8.8–9.8 months), and T12 (11.6–12.7 months).

windows, IBM SPSS Inc, IL USA) and GraphPad Prism 7.0 software was used for Figures (California, USA). Lattice plots were produced using R software version 2.15.2.

RESULTS

Participants

The demographics and characteristics of the study participants are described in **Table 1**. Of 18 mothers, 16 were Caucasian and 2 Asian; 17 mothers were married; 15 mothers completed last year (year 12) of school, 12 indicated various diplomas as further education. Three mothers reported depression and one exhibited hypertension.

All 18 infants were exclusively breastfed at 2 and 5 months and continued to breastfeed at 9 months. Fifteen infants (83%) continued to breastfeed on demand at 12 months. One male infant ceased breastfeeding 8 days before the 12-month appointment, one male infant was being weaned at the time of 12-month appointment, no samples were provided; one female infant stopped at 10 months after birth; these three infants were measured at the time of the 12-month appointment. One male infant was sick and did not attend the last appointment.

Infant % FM ($p < 0.00$) changed significantly with an increase from 2 to 5 months, followed by a decrease at 9 and 12 months.

Changes in HM Glucocorticoid Concentration Throughout the Year

The concentration of cortisol and cortisone in each individual and at each time point is shown in **Figure 1**. The concentration of cortisol ranged between 0.01 and 5.82 ng/ml and cortisone ranged between 2.60 and 13.23 ng/ml (**Table 2**). For all analyzed samples, cortisone was the predominant glucocorticoid at all the four time points. Mean concentrations of cortisol ($p = 0.10$), cortisone ($p = 0.06$) and cortisol/cortisone ratio ($p = 0.39$) did not differ significantly over the period of 12 months.

Relationships Between Maternal Characteristics and HM Glucocorticoids

HM cortisol ($r_s = -0.29$, $p = 0.02$), cortisone ($r_s = -0.27$, $p = 0.03$) and cortisol/cortisone ratio ($r_s = -0.24$, $p = 0.05$) showed associations with maternal height. Furthermore, an overall significant positive correlation was found between HM cortisol/cortisone ratio and maternal BMI ($r_s = 0.33$, $p = 0.009$). Maternal % FM showed no correlation with cortisol ($r_s = 0.21$, $p = 0.09$) and cortisol/cortisone ratio ($r_s = 0.24$, $p = 0.06$).

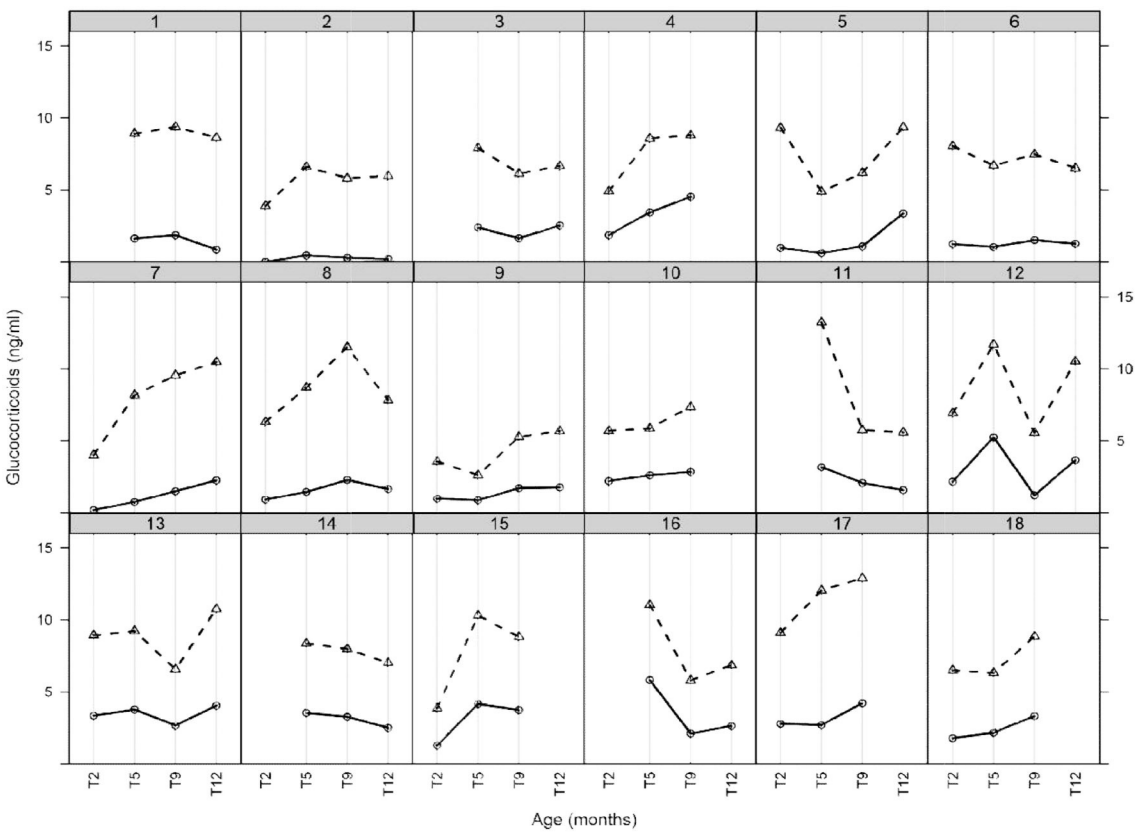

FIGURE 1 | HM glucocorticoids (cortisol and cortisone) during the first 12 months of established lactation. Each box of the lattice plot indicates a single mother milk glucocorticoid profile with symbol (o) representing cortisol and (Δ) representing cortisone. Each solid line indicates cortisol and dashed line cortisone. Time points are categorized as T2 (1.9–2.3 months), T5 (4.8–5.5 months), T9 (8.8–9.8 months), and T12 (11.6–12.7 months).

TABLE 2 | Summary of glucocorticoids in HM samples (n = 63) collected from 18 breastfeeding Western Australian mothers at different stages of lactation (2, 5, 9, and 12 months).

	Mean ± SD (ng/ml)			
Lactation stage (months)	T2.0	T5.0	T9.0	T12.0
Cortisol (ng/ml)	1.52 ± 0.96	2.68 ± 1.65	2.32 ± 1.13	2.18 ± 1.11
Cortisone (ng/ml)	6.19 ± 2.00	8.57 ± 2.72	7.76 ± 2.15	7.83 ± 1.90
Cortisol/cortisone ratio	0.22 ± 0.13	0.30 ± 0.13	0.29 ± 0.11	0.27 ± 0.11

TABLE 3 | Linear regression: associations between milk glucocorticoids and infant characteristics.

Infant factors	Unstandardized β	Standardized β	SE	*p*
Infant percentage fat mass (with cortisol)	0.08	0.24	0.04	0.05
Infant head circumference (with cortisone)	0.21	0.26	0.10	0.03

The regression analysis identified a positive relationship between HM cortisol and maternal height ($p = 0.02$), and maternal BMI ($p = 0.04$). However, no association was found between HM cortisone and maternal BMI ($p = 0.81$) or maternal height ($p = 0.60$).

Relationships Between Infant Characteristics and HM Glucocorticoids

Overall, a weak positive correlation was found between cortisol and infant head circumference ($r_s = 0.25$, $p = 0.05$) and % FM ($r_s = 0.27$ $p = 0.03$). Furthermore, the HM cortisol/cortisone ratio showed a positive correlation with infant % FM ($r_s = 0.34$ $p = 0.01$) and BMI ($r_s = 0.28$, $p = 0.032$), while cortisone showed no significant associations with any of the infant parameters ($p = 0.07$, head circumference, $r_s = 0.23$). The mixed model analysis showed a positive relationship between HM cortisol and infant % FM ($p = 0.008$), and head circumference ($p = 0.05$); and showed no associations with infant length ($p = 0.37$), weight ($p = 0.56$), and BMI ($p = 0.26$). Whereas, cortisone showed a significant positive association with infant head circumference ($p = 0.01$) and showed no association with infant % FM ($p = 1.00$), length ($p = 0.61$), weight ($p = 1.00$), and BMI ($p = 1.0$). Furthermore, cortisol/cortisone ratio showed a significant association with infant % FM ($p = 0.04$) and no associations were found between cortisol/cortisone ratio and infant length ($p = 0.50$), weight ($p = 0.81$), BMI ($p = 0.72$), and head circumference ($p = 0.11$). Follow-up analysis using linear regression (**Table 3**) established that infant % FM and head circumference significantly associated with cortisol (PE+/–SE; $p = 0.05$) and cortisone (PE+/–SE; $p = 0.03$) in HM, respectively.

DISCUSSION

The concentrations of the glucocorticoid hormones in HM, cortisol and cortisone, exhibited unique individual variation at the measured timepoints during the first year on life. Averaged across the sampled cohort, there was no consistent pattern in the HM glucocorticoid concentrations throughout the first year of lactation. However, this study providing evidence that higher concentrations of HM cortisol were positively and significantly related to greater infant adiposity (% FM). Higher concentrations of HM cortisone were positively associated with larger infant head circumferences. These data provide preliminary evidence that HM glucocorticoids exert influence on infant growth and body composition. HM cortisol was correlated with maternal BMI and height, but not HM cortisone, suggesting a more complex relationship between HM glucocorticoids and maternal adiposity.

The composition of HM is influenced by the complex interplay between maternal, infant and diverse environmental factors (10); and also varies depending upon the stages of lactation (34–36). In the current study, it was demonstrated that cortisol, cortisone and their ratio did not significantly change over the first 12 months of lactation. These data further confirm the prior demonstration that HM cortisol remained unaltered between 1 and 12 months of lactations (26). Interestingly, the apparent concentration of cortisol measured in this earlier study (26) ranged between 0.2 and 32 ng/ml, which was on average, 5.5-fold higher than that of the current study. Given the quantitative nature of the current LC-MS technique and the ability to accurately discriminate between differing glucocorticoids, it is then likely that the previously used immunoassay may have exaggerated the abundance, potentially due to antibody cross-reactivity.

In this study, HM cortisol is significantly associated with adiposity (% FM) in infants, with higher levels of HM cortisol being associated with greater infant adiposity (over the first 12 months of lactation). Elevated circulatory cortisol is known to be a potent stimulator of body fat mass gain and the mechanisms are well-described (37, 38). However, majority of these studies have been done in adults with metabolic syndrome and hence require more robust studies on the long term effect of glucocorticoids on the breastfeed infants. A recent study by Hahn et al. (21) provided prior evidence that HM cortisol is the predictor of infant obesity. In this study, infants exposed to higher milk cortisol concentration exhibited reduced infant body mass index percentile (BMIP) at 2 years of age. Furthermore, they reported that the milk cortisol did not predict weight gain, but infants exposed to elevated level of cortisol grew taller, causing the variations in BMIP. However, not much is known about the impact of milk-ingested glucocorticoids, particularly cortisoneThis study did not examine the relationship of HM cortisone. Unlike cortisol, cortisone is the biologically inactive steroid, requiring type 1-11β-hydroxysteroid dehydrogenase (11 βHSD) enzyme conversion into physiologically active cortisol and 11 βHSD type-2 for converting cortisol into cortisone (39). Since cortisone is not secreted in measurable amounts in maternal plasma, 11 βHSD type-2 could potentially be present within the milk or mammary gland. However, the function and mechanisms of this conversion in milk remains largely unknown.

During infancy or early childhood, head circumference is commonly used as an indicator of infant brain size (40) and is also

used as a proxy for infant's neurological development, cognitive function and intracranial volume (41). Numerous prospective studies have shown that chronic prenatal maternal stress during pregnancy influences infant brain development and is associated with smaller head circumference, although the underlying mechanisms are unclear (42–46). Head circumference is an old method of measuring infant brain development, but still is considered reliable. Dupont el al. (47) investigated the predictive value of head circumference during the first year of life on early child development. They demonstrated that post-natal head circumference growth positively predicted gross motor skills as well as behavioral growth at 2 years of age. However, only a few studies have investigated the relationship between maternal perceived stress and infant neurodevelopmental outcomes (48). Studies examining these associations have pronounced mixed results in humans; some found a negative association between maternal stress and infant head circumference (45, 49), while others failed to show any association (43). Chronic prenatal stress disrupts cognitive performance and reduces brain volume in the area related to learning and memory (44). A negative association between prenatal maternal stress and fetal head growth development suggests a key role of maternal stress in regulating fetal head growth (45). Recently the role of glucocorticoids has become evident in infant development (50, 51), and in this current study, we identified that increasing head circumference could be associated with HM glucocorticoids. Although, correlation does not imply causation, hence results should be read with caution.

An association between early glucocorticoid exposure and infant metabolism is complex and regulated by many maternal related factors such as maternal circulating levels. Excessive exposure to both endogenous and exogenous glucocorticoids during pregnancy and lactation have been previously linked to obesity risk factors (52, 53). This study has shown a significant association between maternal BMI and HM cortisol levels. There are several factors that contribute to the plasticity of HM, including maternal BMI, an important contributor to the hormonal profile of HM, further affecting the developmental trajectories of HM fed infants (29). Evidence suggests that individuals with higher BMI are more likely to have an increased level of circulating cortisol (54, 55). This study is the first to demonstrate an association between maternal BMI and HM cortisol concentration. This is in accordance with previous studies that report obesity or higher BMI to be an indicator of increased circulatory or saliva cortisol level (56, 57).

A limitation of the current study was the absence of maternal plasma samples. Therefore, this study is unable to report on possible relationships between maternal hypothalamic variabilities and the HM glucocorticoids. Furthermore, the sample set was small and predominately from Caucasian women. Further studies examining HM hormonal profiles and infant outcomes, at different stages of lactation, will further elucidate the possibilities of lactocrine programming.

In conclusion, HM cortisol and cortisone demonstrated unique variability in each women, but on average remained constant throughout the first 12 months of lactation. HM cortisol and cortisone were positively correlated with infant head circumference and % FM during the first year of life. This current research points to the need to better understand the determinants of HM glucocorticoids regulation. Yet the implications the HM glucocorticoids function to regulate infant metabolism, appetite and behavior suggest that further exploration is required to better understand how ingested hormones can mediate these peripheral actions.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The University of Western Australia Human Research Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SP conducted the analyses, drafted the initial manuscript, reviewed, and revised the manuscript. All authors were responsible for the idea conception, approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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REVIEW
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Nutritional and Non-nutritional Composition of Human Milk Is Modulated by Maternal, Infant, and Methodological Factors

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Human milk (HM) is dynamic and shows a high inter- and intra-individual variability. To characterize HM with precision, it is necessary to understand the factors that modulate its composition. The objective of this narrative review is to summarize the maternal, infant and methodological factors that affect HM composition. We searched SCOPUS and PubMed databases for articles related to factors that are known to or could potentially influence HM composition and volume across lactation periods. Our comprehensive review encompasses various maternal-, infant-related, and methodological factors that modulate aspects of HM composition including macro- and micronutrients, vitamins and minerals, as well as volume. The most profound changes were observed in HM lipids and lipophiles. Evidence exists for many of the infant-related factors known to affect the nutritive and non-nutritive components of HM (e.g., birth weight, gestational age, infant age/stage of lactation). In contrast, less is known with respect to maternal factors; where there is either limited research or conflicting evidence (e.g., maternal lifestyle, obstetric history, medical conditions), except for the mother's diet, for which there is a relatively well-established understanding. Equally, although many of the methodological factors (e.g., HM sampling, handling and analytics) are known to impact HM composition, few studies have investigated this as a primary outcome, making it an important area of future research in HM. Here we propose a systematic capture of numerous maternal- and infant-related characteristics to facilitate associative comparisons of HM data within and across studies. Additionally, it would be prudent to standardize the methodological aspects known to affect HM composition in analytics, not only for HM lipids and lipophiles, but also for those nutrients whose variability is yet less well-understood. Defining the factors determining HM composition with accuracy will open perspectives for maternal intervention to optimize milk composition for specific needs of infants.

Keywords: human milk, lactation, maternal, infant, human milk sampling, standardization, human milk composition

INTRODUCTION

Breastfeeding i.e., human milk (HM[1]) feeding either from the infant's own mother, or wet nurses as in the past, was and continues to remain the feeding norm for new-born infants. Benefits of breastfeeding for both infants and mothers, in short- and long-term are well-documented (1–5). A recent publication indicates a protective effect of breastfeeding against childhood infections and malocclusion, increases in intelligence, and probable reductions in overweight and diabetes later in life (6). Mothers may also benefit from breastfeeding in that risk of breast, uterine and ovarian cancer is reduced, and post-partum weight loss is promoted (6). With multiple benefits of breastfeeding, it is no surprise that characterization of HM composition is of increasing interest in order to gain insights into the underlying factors that contribute to the benefits of breastmilk.

HM is a highly complex, and dynamic biological fluid rich in nutritive (e.g., lipids, proteins, carbohydrates, fatty acids, amino acids, minerals, vitamins, trace elements, etc.) and non-nutritive bioactive components (e.g., cells, Ig, cytokines, chemokines, hormones, growth factors, glycans, mucins, etc.). It provides protection against infection and inflammation, and contributes to immune maturation, organ development, microbiota colonization and overall infant health (7–9).

It is hypothesized that HM is tailor-made by each mother to meet the nutritional needs of her growing infant; plasticity of HM composition may be key to early infant growth and programming of health in later life. Studying HM composition is essential to not only understand the nutritional needs but also to correlate with other developmental outcomes of infants (e.g., neurodevelopment, immune development, and gut maturation). Understanding the associations between HM composition and developmental outcomes can also lead to strategies for modifying maternal nutrition in cases where needed and to adapt fortification strategies for preterm infants.

While there exist published reviews in which a selection of factors that influence the composition of HM is described, to our knowledge no review considers the numerous aspects that affect the composition and volume of HM. With our narrative review we aim to collate the most relevant factors that influence nutritional and non-nutritional composition of HM, namely both maternal- and infant-related factors. In addition, we highlight methodological aspects that affect the quantification of HM components in research settings, which in turn leads to a set of scientific recommendations for HM sampling, handling, and analytical characterization when embarking on research in this field. On the one hand, we attempt to be as comprehensive as possible. On the other, it would simply be too much for one paper to provide an in-depth review of all topics that modulate HM composition and volume. Therefore, the purpose here is to identify and focus on the most important factors including maternal diet, supplementation, galactagogues, maternal anthropometry, obstetric history, socio-demographic factors, medical conditions, as well as infant-related factors such as infant birth weight and sex. Temporal changes in HM volume and composition are also included. In addition, methodological factors affecting the quantification of HM components are assessed. Other factors are deprioritized but summarized and listed as further reading.

Abbreviations: HM, human milk; PUFA, polyunsaturated fatty acid; LA, linoleic acid; ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; FA, fatty acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; GPC, glycerylphosphorylcholine; TMAO, trimethylamine N-Oxide; BMI, body mass index; Ig, immunoglobulin; EGF, epidermal growth factor; PCBs, polychlorinated biphenyl; PCDDs, polychlorinated dibenzo-p-dioxins; PCDFs, polychlorinated dibenzo-furans; GDM, gestational diabetes mellitus; IL, interleukin; MFG, milk fat globule; HMO, human milk oligosaccharides; b-FGF, fibroblast growth factor; IGF-1, insulin-like growth factor-1; TN, total nitrogen; NPN, non-protein nitrogen; HMA, human milk analyser.

[1]2 FL, 2′-fucosyllactose; i, 3′-Fucosyllactose; 3 SL, 3′sialyllactose; AA, Amino acid; BCA, bicinchoninic acid assay; EGF, epidermal growth factor; FM, fat mass; b-FGF, fibroblast growth factor; GDM, gestational diabetes mellitus; GPC, glycerylphosphorylcholine; HM, human milk; HMO, human milk oligosaccharides; IR, infrared; IGF-1, insulin-like growth factor-1; MFG, milk fat globule; NPN, non-protein nitrogen; PCBs, polychlorinated biphenyl; PCDFs, polychlorinated dibenzo-furans; PCDDs, polychlorinated dibenzo-p-dioxins; TN, total nitrogen; TMAO, trimethylamine N-oxide.

METHODOLOGY

Literature Search

We conducted a narrative literature review and search using SCOPUS and PubMed database with predefined keywords for all scientific literature related to factors that are known to or could potentially influence HM composition and volume across lactation.

An initial search was performed limited to titles, key words, and abstracts to assess the potential yield of the search strategy. Based on the results, search terms were modified (e.g., wild characters) or additional terms were included. A subsequent search included MeSH terms. The search terms were grouped into 2 different concepts, which were combined with Boolean term "AND." Terms within each concept were combined with the Boolean term "OR."

Concept 1: human milk, breast milk, mothers' milk, mothers' own milk, colostrum

Concept 2: maternal/mothers' diet, supplementation, anthropometry, smoking, alcohol consumption, coffee consumption, physical activity, breastfeeding frequency, parity, mode of delivery, cesarean section, age, socioeconomic status, psychology, drugs, galactagogues, infant sex/gender, gestational age at birth, small for gestational age, low birth weight, foremilk/midmilk/hindmilk, preterm, feeding time, type of expression, storage temperature/length, milk processing/pasteurization.

The search was limited to English language abstracts and articles only.

Selection Criteria

We did not eliminate any kind of study design for initial screening as long as it met search criteria. Articles include original research, literature review and conference abstracts/papers/presentations that were published up to December 2018. After a final selection by reading the abstract of each article, 260 relevant papers were included.

TABLE 1 | Maternal factors affecting nutritive and non-nutritive components of human milk.

Factors	Effect on nutritive or non-nutritive components of HM	Evidence	References
Dietary or supplementary intake	Changes in maternal intake modify amino acids, (n-3) FA, iodine, selenium, thiamine, riboflavin, niacin, vitamin B6, vitamin B12, choline, vitamin C, vitamin A, provitamin A carotenoids, vitamin D, vitamin K	Evidence probable	(10–67)
	Changes in maternal intake do not modify calcium, magnesium, copper	Evidence probable	
	Changes in maternal intake do not modify HM volume, energy, macronutrients, phosphorus, potassium, sodium, chromium, cobalt, fluoride, iron, manganese, molybdenum, zinc, folate, non-provitamin A carotenoids, vitamin E	Inconsistent or lacking	
Anthropometry	The higher the BMI the higher the concentration of SFA, n-6/n-3 FA ratio, leptin in HM	Evidence probable in high-income countries Lacking in low-middle income countries	(68–82)
	The higher the BMI the higher the HM intake by the infant	Inconsistent or lacking	
Parity	Higher parity increases amounts of proteins, lipids, and improves FA profile	Inconsistent or lacking	(83–89)
	Multiparous women show increased concentrations of iron and Ig	Inconsistent or lacking	
Mode of delivery	Affects iodine, microbiome, IgA, or bacterial diversity	Inconsistent or lacking	(88, 90–93)
Age	Influences the content of lactose, lipids, protein, TN, NPN, Na, K, Ca, P, Cu, colostrum Ig	Lacking	(83, 84, 86, 87, 94, 95)
	Maternal age is negatively correlated with FA profile and Zn	Lacking	
Socioeconomic status	Socioeconomic status is associated with lipids, proteins, IgA, and FA profile	Inconsistent or lacking	(83, 95–98)
Medical conditions	Maternal GDM delays the initiation of lactation or reduces hormones	Inconsistent or lacking	(99–127)
	Maternal post-partum depression is associated with a shorter duration of lactation and perceived HM production	Inconsistent or lacking	
	Celiac disease reduces immune protective compounds (TGF-β1, sIgA) and bifidobacteria	Lacking	
	Allergies affects levels of interleukins, growth factors, beta-casomorphin, selective proteins, pro-inflammatory markers, cytokines	Inconsistent or lacking	
	Mastitis results in higher levels of minerals, MFG, and chlorides as well as higher catalase activity and increased concentration of IL-6	Lacking	
	Mastitis results in lower contents of lactose, lipid, protein, and casein	Lacking	
Use of galactagogues	Increase HM volume	Inconsistent or lacking	(63–66)

RESULTS

Factors that impact HM composition and/or volume are summarized into the following two categories:

1. Maternal and infant characteristics affecting HM components
2. Methodological parameters affecting the quantification of HM components

The resulting information leads to a set of scientific recommendations for reproducible HM analysis in interventional as well as observational studies (see Methodological factors affecting the quantification of HM components in a research setting).

Maternal Factors Affecting HM Volume and Composition

Maternal Diet

Maternal diet is an important factor that influences the volume and composition of HM. Some components have been studied extensively, while others require further research to draw strong conclusions. The results of our analysis are summarized in **Table 1**. Furthermore, a systematic analysis of the influence of maternal diet on HM composition, micro- and macronutrients, emphasized the relation of fatty acid intake, the intake of fat-soluble vitamins, vitamin B1, and vitamin C to their content in HM (10).

Effects of maternal diet on macronutrient content in HM

The macronutrient composition of HM is relatively constant across populations despite variations in the nutritional status of the mother (7). The effect of maternal energy intake on HM volume has shown conflicting results. Some studies showed that provision of additional calories (11, 128), energy restriction (12), or fasting (13) had no impact on HM volume, while others showed that a low calorie diet (1,200 kcal per day for 3 days) (14) or low energy intake (15) resulted in reduced HM production. In terms of macronutrient composition, an isocaloric high fat diet (16) and increased dietary consumption of proteins (17) and carbohydrates (18) demonstrated an increased HM fat concentrations at different stages. No significant differences in energy content of HM were found up to 12-weeks post-partum supplemented with a commercially available product, despite the higher energy intake (19). The same study revealed

a greater volume of HM in mothers with a low mid upper arm circumference (<24.1 cm) following supplementation. Several reviews have highlighted the population differences in HM fatty acid composition and the influence of maternal diet on levels of PUFAs [including HM linoleic acid (LA), α-linolenic acid (ALA), and DHA] (20–22). A study provided supporting evidence that the quality of fatty acid intake during pregnancy and lactation is important (23). In this study the abundance of n-6 fatty acids (FA) and low n-3 FA, was reflected in the fatty acid composition of HM, in as such that DHA concentrations were significantly reduced particularly during lactation compared to pregnancy, while arachidonic acid (AA) did not follow the same development. On the contrary, women who consumed 2 portions of salmon per week from 20 week of pregnancy until delivery showed higher proportions of EPA (80%), DPA (30%), and DHA (90%) on day 5 post-partum (24). The impact of omega-3 supplementation during pregnancy and/or lactation on HM FA is well-studied: a systematic review found a positive relationship between the consumption of omega-3 sources and their concentration in HM, despite differences in the methods used, timing of supplementation, source of omega-3 source used, and the sample size (25). Supplementation with fish oil starting at 20 weeks of pregnancy until delivery not only increased the long-chain omega-3 FAs in HM during early lactation (up to 6 weeks post-partum), furthermore DHA levels at day 3, 6, weeks and 6 months were positively associated with infant DHA status at 1 year (26). Supplementing lactating women 4–6 weeks post-partum with either 200 mg or 400 mg DHA for 6 weeks with usual diets led to a 50 and 123% respective increase in HM DHA levels (27).

In terms of carbohydrate type, a randomized crossover study revealed that the consumption of a high-fructose corn syrup–sweetened beverage increased concentrations of fructose in HM, which stayed elevated for 5 h (28). No such effects were found for glucose or lactose. The consequences of habitual consumption of high-fructose sweetened beverages for early development need to be further investigated, but evidence exists that fructose in HM is positively associated with infant's body composition (29).

Effect of maternal mineral supplementation on mineral content of HM

Iodine supplementation (75–400 mg iodine/day) increased HM iodine concentrations (30–32), although a lower dose (75 or 150 μg/d) through either supplementation (31) or fortification (33) may be insufficient to ensure adequate iodine status in women or their infants. Equally, administering a single dose of 400 mg iodine as oral iodized oil to mothers may be an effective strategy to provide adequate iodine to their infants through HM for at least 6 months compared with direct supplementation to infants (30). The results of Mulrine et al. where iodine concentrations in HM were 1.3 and 1.7 times higher in women supplemented with 75 and 150 μg per day would also suggest a dose-response relationship (31). A systematic review of epidemiological and clinical data suggested that a high dose as well as daily iodine supplementation were effective in increasing iodine concentrations in HM (34). The same authors also concluded that iodine concentrations of around 150 μg/l during the first 6 months of lactation are enough to meet infants' needs. Selenium (Se) supplementation at a dose of 20 μg/day, approximating the quantity secreted into HM, (35) or 200 μg/day (36) increased HM selenium concentrations accompanied by an increase in infant Se intake. It is known that HM concentrations of minerals such as zinc and iron are not largely altered by deficiency or excesses in the maternal diet as these have well-regulated homeostatic processes (37). Zinc supplementation studies (10–50 mg/day) provided inconsistent findings, with some reporting an increase or prevention in decline in HM zinc concentration compared with placebo (38), while others did not find an effect of zinc supplementation (39, 40). Observational studies show reduced levels of iron in the HM of anemic mothers (41) and iron supplementation among women with low baseline levels of iron, was shown to increase HM iron concentrations (42). However, studies among healthy lactating women have failed to demonstrate an association between iron intake of the mother and HM iron concentrations (37, 43). There are physiological changes that happen during lactation, such as changes in calcium and bone metabolism, that provide sufficient calcium for HM production independent of maternal calcium supply in populations where calcium intakes are close to current recommendations (44). Furthermore, dietary nutrient consumption had no strong association with calcium concentrations in HM of three different ethnic groups in New Zealand (45). In this study, calcium intake of the mothers varied from 736 to 1,041 mg/day, but had no significant effect on the calcium concentration in HM.

Effect of maternal vitamin supplementation on vitamin content of HM

Maternal supplementation of B vitamins during lactation is known to rapidly increase their concentrations in HM as reviewed extensively Allen et al. (46). Evidence suggests that HM concentrations of thiamine, similar to those observed in well-nourished mothers, can be maintained by maternal thiamine intake of 2 mg/d (47). The concentrations of vitamin B6 in HM (ranging from 0.89 to 1.31 nmol/L) appear to be saturated at an intake of 2.5 mg/d of vitamin B6 (48, 49). Vitamin B12 supplementation (50 μg/d) was shown to maintain elevated concentrations of B12 in HM only during the supplementation period (6-week post-partum) (50) and the concentrations observed were much lower than those reported with a higher dose of 250 μg/d dose (97 vs. 235 pmol//L) (51). Choline supplementation of 750 mg/d increased HM concentrations of free choline, betaine, and phosphocholine (52), while a higher dose (930 mg/d) resulted in significantly higher concentrations of phosphocholine and glycerylphosphorylcholine (GPC), the main choline metabolites in HM as well as HM trimethylamine N-Oxide (TMAO), which is essential for maintaining osmotic balance and glycine concentrations (critical for production of glutathione that appear limiting in early development) (53). Similarly, higher lutein and zeaxanthin concentrations in HM have also been observed with lutein supplementation of 6 mg/d (140% higher) and 12 mg/d (250% higher) compared with the placebo (54). Further evidence comes from a study with Korean mothers, where a higher dietary intake of lutein was associated

with higher lutein content in HM (55). In this study, the mean intake of lutein was 4.7 mg/day, with a range from 0.64 to 17.05 mg/day, while the mean lutein concentration in HM was 3.5 μg/dl, with a range from 0.5 to 31.3 μg/dl. Although no intervention studies have been reported so far, observational data suggest that the concentrations of biotin in the HM decreased over the course of lactation despite an intake of 57 mg biotin/d which is 63% higher than the adequate intake of 35 mg biotin/d, suggesting that a low intake of biotin may accelerate the decrease (56). There is strong evidence that retinol or beta carotenoid concentrations in colostrum (1–5 days) or mature (>14 days) HM are increased through supplementation of vitamin A during lactation either as retinyl palmitate (658–7,218 μg per day), as multivitamins containing β-carotenoids (providing 90 mg/d beta carotene) or as part of a diet enriched with vegetable oils like red palm oil (providing 90 mg/d beta carotene), soybean oil or sunflower oil (22). The same is true for a higher vitamin A consumption (range of 50–7,205 μg retinol activity equivalents), which led to higher, but non-significant, retinol concentrations (57). A strong positive association was demonstrated between maternal vitamin D intake during exclusive breastfeeding and infant serum 25-hydroxyvitamin D levels (58), although doses at the current recommended daily intake of vitamin D may not be sufficient for adequate transfer from mother to the breastfed infant (58) and supplementation during lactation with 4,000–6,400 IU/d of vitamin D may be needed to prevent vitamin D deficiency in the mother and her breastfed infant (59). The mode of supplementation needs to be taken into account, as a randomized controlled study demonstrated that a single bolus led to a higher production of vitamin D, compared with a daily dose (60), and levels remained elevated for at least 28 days. Among women with adequate folate status, HM folate secretion was shown to reach a maximum threshold and was not affected by the maternal folate status, except in clinically folate-deficient mothers (46). Maternal supplementation with alpha-tocopherol was shown to increase the vitamin E concentrations in colostrum, although the evidence is limited (61).

Galactagogues

Adequate lactation is fundamental for the (premature) infant's health, hence there is an increasing interest in galactagogues. Galactagogues are substances that have the potential to assist in various stages of milk production, such as initiation, continuation, or augmentation (62). Pharmaceutical agents, such as domperidone, or more recently herbs such as silymarin-phosphatidylserine and galega are widely used (63, 64). Many herbal concoctions have been proposed as galactagogues around the world. However, there is more anecdotal evidence rather than scientific rigor. There is a huge cultural as well as regional aspects on what is considered or promoted as a galactagogues. From many herbal galactagogues, fenugreek has most number of clinical trials reported in the literature and a recent network meta-analysis demonstrated its efficacy against placebo (65). Moreover, a study reported that a herbal tea containing stinging nettle, among other herbs, increased milk production in 36 mothers with premature infants by 80%, a significant increase when compared with the placebo and control group (66). Overall, the quality of galactagogue literature was found to be poor and the field would benefit from well-designed double blinded and randomized controlled studies.

Maternal Anthropometry

Influence of maternal weight/body composition on HM lipids and fatty acid profile

HM of overweight women is reported to contain higher amounts of SFAs, lower amounts of n-3 FAs, a lower ratio of PUFAs to SFAs and a higher ratio of n-6 to n-3 FAs, compared with normal weight women, even after adjusting for maternal diet (68). A study with 80 women reported a significant maternal age and body mass index (BMI) interaction with calorie and fat. BMI alone was significantly associated and protein content (69). Calorie and fat contents were found to be lower in overweight mothers in their 20s but higher in older, overweight mothers. The reason for this unique finding is not fully understood. The overall nutritional status of the mother and the stage of lactation may be important factors that deserve attention while studying the association between maternal BMI and HM macronutrient concentrations. To exemplify this, studies among well-nourished lactating women have failed to demonstrate any association between maternal fat stores and HM lipids (70), while those conducted among marginally nourished populations showed a positive relation both in early (<90 d) and late (≥90 d) lactation (71, 72).

Influence of maternal weight/body composition on volume of HM

Maternal body composition not only influences the composition of HM in terms of its macronutrient composition, but is also associated with the volume of HM transferred to infants when assessed using the "dose to the mother" deuterium dilution method (73). This was shown in infants from Kenya, where HM intake was positively correlated to maternal triceps and mid upper arm circumference during pregnancy (74). In contrast, among low-income Honduran women maternal anthropometric status was not found to be a significant predictor of HM volume or infant energy intake between 4 and 6 months of age after accounting for confounding factors such as birth weight and milk energy density. This highlights the ability of the infant to self-regulate intake in response to milk energy density and infant weight (75). Furthermore, studies reported positive associations between maternal BMI and HM leptin levels (76–79). One study found higher maternal %FM associated with higher leptin but not adiponectin concentrations (80). The same authors hypothesized that HM adiponectin levels are less likely to be impacted by maternal adiposity as the majority of HM adiponectin may be synthesized and controlled by the mammary gland. Others reported positive associations between HM leptin and adiponectin concentrations with infant weight gain (77, 78, 81) as well as HM adiponectin concentrations and fat mass in the infants up to 2 years (82).

Maternal Obstetric History

The influence of parity on HM composition

Parity has been found to be an independent factor influencing proteins and lipids content (83) and FA profile (84). However,

findings are not consistent in the literature. Bachour et al. (83) reported an increased lipid concentration of HM associated with higher parity among Lebanese mothers. In contrast, among rural Gambian women, the amount of HM fed to the infant and the proportion of endogenous FAs in HM was markedly lower among those who had borne 10 or more children, implying an impairment of the ability to synthesize HM FAs *de novo* for high parity women (85). In terms of mineral content, increased amounts of iron were found in multiparous women (86). Islam et al. (87) did not find an association between parity and colostrum Ig concentration (including IgA, IgM, IgG, and peripheral immune cells) among women from Bangladesh, yet another study showed, increased IgA and IgM in colostrum of primiparous compared with multiparous Brazilian women (88). It is further hypothesized that a mother's breastfeeding of higher birth-order infants would be exposed to a wider array of organisms from their other children, and these could affect milk cytokine levels (89).

The influence of mode of delivery

Mode of delivery, including cesarean and vaginal delivery, was shown to affect protein content in colostrum (90), HM choline level (91), HM iodine concentration (92) and IgA concentration (88). Some data also suggested that vaginal delivery is associated with higher protein content in colostrum when compared with cesarean delivery (90). Ozarda et al. (91) found higher levels of choline in HM after cesarean vs. vaginal delivery. Iodine concentration in the transitional milk was found to be higher in women with cesarean section (349.9 μg/kg) in comparison with those with vaginal delivery (237.5 μg/kg, $p < 0.001$) (92). Higher IgA concentration was found in colostrum of women having undergone cesarean section rather than vaginal delivery, because of the occurrence of labor together with surgical stress (88). However, in a multi-center study, mode of delivery was not found to be a significant factor in IgA concentrations in HM (93). The effect of the mode of delivery on HM IgA concentration is suggested to be confirmed in larger cohorts (89). While we have started to observe the differences in HM composition between groups, further studies should focus on the implications of such differences on the health outcomes of the infants.

Maternal and Socio-Demographic Factors

Maternal age

The potential influence of maternal age on HM composition is still being debated. Early studies indicated that the lactose, fat, total nitrogen, protein nitrogen, non-protein nitrogen, sodium, potassium, calcium, and phosphorous concentrations differed little between adolescent and adult breastfeeding mothers (94). Likewise, maternal age was not related to colostrum Ig concentration (including IgA, IgM, IgG, and peripheral immune cells) (83, 87), milk lipids (83, 95), proteins (83), and copper contents (86). In contrast, Antonakou et al. reported maternal age as an independent factor, demonstrating a strong negative association between maternal age and MUFAs, including oleic acid proportions particular during the first months of lactation (84). Also, Silvestre et al. reported lower zinc contents in HM from older women when compared with HM from younger women (86).

Maternal socioeconomic status

There is no consistent evidence about the influence of maternal socioeconomic status on HM lipid contents. Rocquelin et al. did not find an association between socioeconomic status, e.g., mothers' education/occupation and HM lipid content among Congolese mothers of 5 month old infants (95). Bachour et al. found a notable but not statistically significant association between maternal residential area and milk lipids, proteins and IgA levels (83). In contrast, Al-Tamer and Mahmood demonstrated an effect of maternal socioeconomic status of lactating mothers in Iraq on HM lipid content, TG and FA composition, especially the proportions of long-chain omega-3 FAs decreased with decreasing socioeconomic status (96). Similarly, a study in low-income Indian women demonstrated an association of socioeconomic factors, particularly maternal education, with HM composition in as such that a higher education resulted in lower concentrations of SFAs and PUFAs (97). In Chinese women a higher education was positively associated with concentrations of carotenoids and tocopherol in HM (98).

Geographical location

There is consistent evidence in the literature that HM FA composition varied according to geographical location, and variation was frequently attributed to the differences in maternal diet (129–133). For example, Chulei et al. reported different concentrations of DHA and AA: DHA ratio in HM in five distinct geographical regions (pastoral, rural, urban 1, urban 2, and marine) of People's Republic of China (130). Roy et al. revealed differences in HM n-6 and n-3 PUFAs between urban and suburban mothers in Bengali, India, and contributed the rural and urban differences to the mothers' alimentary habits (134). A study comparing the levels of omega-3 and omega-6 PUFAs in HM of Swedish and Chinese women demonstrated a more favorable balance of those FAs in Swedish women (135). In addition to lipid content, mineral composition of HM, namely phosphorous was found to vary among three different regions among 444 Chinese lactating women (92). Oligosaccharide profiles in colostrum varied between Italian and Burkinabe women, in as such that in contrast to Italian women, the Burkinabe women had higher concentrations of 2 fucosyllactose and lower concentrations of lacto-N-fucopentaose in colostrum (136). The relevance of these findings is not clear. Like FAs, growth factors vary according to geographical location. For example, 228 breastfeeding mothers from 3 regions in People's Republic of China revealed significant differences in the content of epidermal growth factor (EGF) and TGF-α content in HM by region (137). Differences in diet may explain some of the findings as the concentration of EGF in HM significantly decreased with increasing intake of proteins, total energy, vegetables, fruits, soy products and dairy foods, while the TGF-α content in HM significantly increased with increasing intake of carbohydrates and dairy products and decreased with increasing intake of proteins and meat. Overall, we hypothesize

that geography is a proxy factor for underlying factors that drives the differences in HM composition. The major factor that remains common to a cohort in geography such as diet or life style are usually the underlying causes of demonstrated differences in HM composition.

The influence of maternal exposure to toxins on levels of toxins in HM

Human milk of mothers with different childhood exposures differs significantly. It might be possible that exposure of the mother during her own infancy primes her intestinal immune cell development in as such that when immune cells migrate from the maternal intestine to the mammary gland, results in altered HM composition. It was reported that women who were breastfed during infancy and grew up on the Baltic coast of Sweden had high levels of polychlorinated biphenyl (PCBs) and polychlorinated dibenzo-p-dioxins (PCDDs)/polychlorinated dibenzo-furans (PCDFs) in HM. Exposure early in life to HM and consumption of contaminated fish from the Baltic sea were the reasons for high toxic level of PCBs and PCDDs in their HM (138). Also, toxic metal contamination in HM is of concern (139). The authors found, for example, arsenic in 64% of HM samples in Lebanese women and identified an association with cereal and fish intake. In general, inter-country studies reported varying levels of perfluorinated carboxylic acids in HM samples from Japanese, Chinese and Korean mothers, with the highest levels in Japanese mothers (140). More specifically, a strong positive association of perfluoroalkyl substances in HM with maternal age, BMI and parity was reported from an analysis of HM from 128 Korean mothers (141). The same authors reported the consumption of snack, milk, and eating-out frequency to be associated with increased levels of perfluoroalkyl substances.

Maternal Medical Conditions

Effects of gestational diabetes mellitus (GDM) on composition of HM

Maternal prenatal metabolic profile influences the composition of HM (99). It was shown that mothers with diabetes experienced lactogenesis II later than healthy mothers (100). A relatively small study showed that levels of ghrelin, an appetite-stimulating hormone, were lower in HM of early lactation of mothers with GDM as compared with mothers without GDM, but that ghrelin levels were restored to normal in mature HM (101). These data need to be confirmed in a larger group of women. Moreover, mothers with a higher pre-pregnancy BMI combined with GDM or lower insulin sensitivity had higher insulin concentrations in mature HM compared with normal glycaemic mothers (102). Galipeau et al. found that the presence of gestational diabetes increased the risk of an elevated HM sodium level at 48 h after delivery (127). However, Klein et al. did not find any significant differences in HM free AAs content between GDM and healthy women, regardless of it being colostrum or mature HM (103).

Influence of celiac disease on composition of HM

In comparison to healthy mothers, HM of mothers with celiac disease was found to have a reduced abundance of immune protective compounds (TGF-β1 and sIgA) and bifidobacteria (104). On the other hand, HM of 42 mothers with celiac disease who followed a gluten-free diet showed no difference in anti-gliadin antibodies compared with HM from 41 mothers with a normal, gluten-containing diet (105). This suggests that rather than diet, the immunological memory defines the presence of these antibodies in HM.

Influence of maternal atopy/allergy on composition of HM

The association between maternal atopy/allergy and HM composition is not consistent in the literature. Higher IL-4, IL-5, IL-13 (106), and beta-casomorphin-5 (107) in colostrum, and higher levels of IL-4 (108), lower levels of TGF-β1 (109, 110), TGF-β2 (111), IL-10 (108) and proteome (including protease inhibitors and apolipoproteins) (112) in HM were found in women with history of allergic disease compared with non-allergic women. Higher levels of sCD14 were found in mothers with a positive vs. negative allergic history (113). On the contrary, Marek et al. (109) did not find any difference in IL-4 and IL-10 levels in HM between allergic and non-allergic mothers. Likewise, no differences were reported in pro-inflammatory markers and cytokine concentrations (111, 114, 115) and FA composition (111) in HM between allergic and non-allergic mothers. A study among Danish mothers with atopic dermatitis, mothers with other types of atopy, and non-atopic mothers reported that the HM FA composition was not affected (116). In contrast, women with a combination of eczema and respiratory allergy had lower HM levels of several PUFAs (AA, EPA, DHA, and DPA), and had a lower ratio of long-chain n-3 PUFAs/n-6 PUFAs, even though the fish consumption was not different between groups (117). Their PUFA levels differed not only from that of healthy women, but also from that of women with only respiratory allergy, who had a FA pattern like that of healthy women.

Mastitis

Early evidence indicated that HM of mothers with mastitis showed a higher content of minerals, chlorides, and catalase activity, and lower levels of lactose, fat, total proteins, and total casein fractions, in comparison with HM from healthy women (118). This observation is likely because mastitis inflammation can produce pro-inflammatory cytokines and damage the milk fat globule (MFG). Mizuno et al. found that HM of mothers with mastitis contained larger MFG and higher IL-6 levels in the diseased breast than HM from the healthy breast (119). The same authors reported that this difference was larger if accompanied by systemic symptoms of mastitis (fever/malaise). In a separate study, Buescher and Hair reported that HM of mothers with mastitis had the same anti-inflammatory components and characteristics of HM from healthy mothers; although elevation in selected components/activities was observed (TNFα, soluble TNF receptor II, and IL-1RA and bioactivities that cause shedding of soluble TNF receptor I from human polymorphonuclear neutrophils) (120). These components may help protect the nursing infant from developing clinical illness due to intake of HM from mothers with mastitis.

TABLE 2 | Further reading for factors with potential impact on human milk.

Factors potentially modulating HM composition and volume	Suggested reading
Use of medication	(142)
Hormones	(143)
Stress	(144)
Microbiome	(145–148)
Ethnic groups	(45, 149)
Infant suckling time	(150, 151)
Smoking	(83, 152–155)
Alcohol consumption	(156–158)
Coffee consumption	(159–162)
Physical activity	(163–171)
Breastfeeding frequency	(127, 150, 151, 172)
Immunological composition	(89, 173)
Drug use	(142, 159)
Synbiotics	(67)

Post-partum depression

About 85% of women experience a so called "post-partum blues," which is transitional and lasts ∼2–3 weeks after birth (121). The "blues" does not resolve in some women, and about 10–20% of new mothers experience post-partum depression (122). Post-partum depression is frequently found to be related with a shorter breastfeeding duration (123, 124). Currently, there are no randomized controlled trials to demonstrated causality. A population-based prospective cohort study showed that maternal depression was associated with maternal perceptions of insufficiency of HM but not actual milk quantity (125). This finding was supported in lactating mothers of preterm and term infants, where the perceived mood states had no apparent effect on HM volume (126).

Other Maternal Factors Influencing HM Composition and Volume

For the sake of greater focus on the most relevant factors influencing HM composition and volume, this chapter lists but not discuss in detail other topics that could be considered for planning and conducting research in this area but are not discussed in detail. Instead, further reading is proposed (see **Table 2**). Indeed, many factors have significant impact on human milk composition, however, how those differential composition impacts infant outcomes should be a topic of future studies.

Infant-Related Factors Affecting HM Volume and Composition

The main factors have been summarized in **Table 3**.

Infant Birth Weight

A positive relationship has been suggested between birth weight and HM intake attributed mainly to increased sucking strength, frequency or feeding duration (70). In the DARLING study, weight at 3 months and total time nursing were positively associated with HM intake at 3 months, while no maternal variable (such as age, parity or anthropometric indices) was significantly correlated with intake, volume extracted, or residual milk volume, indicating that infant demand was the main determinant of lactation performance (174). In contrast, no differences were observed in absolute and relative HM transfer volumes and HM zinc concentrations of small for gestational age vs. appropriate for gestational age infants born to low-income, marginally nourished Bangladeshi mothers (175).

Infant Gestational Age at Birth and Term vs. Preterm Delivery

A meta-analysis of 41 studies indicated significantly higher concentrations of "true protein," excluding the non-protein nitrogen, in the first few days post-partum. Lower lactose concentrations (in the first 3 days and some later time points) were found in preterm compared with term HM, although no differences were observed for fat and energy content (177). Compared with term HM, preterm HM was shown to have a highly variable human milk oligosaccharides (HMO) content and was richer in glycosaminoglycans. The latter allows HMOs to competitively bind to pathogens and prevent them from adhering to the enterocytes (178). With respect to mineral content, preterm compared with term HM is reported to have higher concentrations of copper and zinc that decline across lactation and lower concentrations of calcium that remains constant over lactation (178). HM from preterm mothers was shown to have higher levels of IgA, IL-6, EGF, TGF-β1, and TGF-β2 in colostrum compared with term HM (179). Ozgurtas et al. found higher levels of vascular endothelial growth factor, basic fibroblast growth factor (b-FGF) and insulin-like growth factor-1 (IGF-1) in HM from the mothers of preterm infants (180). Similarly, preterm delivery also affects hormone concentrations in HM. In a study with 40 lean mothers, those of the preterm infants showed higher concentrations of obestatin and higher expressions of ghrelin mRNA in mammary epithelial cells, however the latter did not result in higher ghrelin concentration in HM (181). It may be speculated that the synthesis of these peptides and their transport from maternal circulation could contribute to the finding that the transcript level of the gene does not correspond to the peptide level in HM. While the HM of mothers of preterm babies seemed to have a higher concentration of immunological markers, there is conflicting evidence on the ability of gestation age to influence HM PUFA composition. One study showed lower levels of EPA and DHA in HM from mothers of preterm infants (182), while others did not find any association (183). Limited data showed that gestation age may influence HM minerals composition. For example, Ustundag et al. reported lower zinc levels in HM from mothers of premature infants with a similar trend seen in colostrum copper levels (184).

Sex of the Infant

The sex of the infant may also be one of the determinants of HM volume and composition of HM, for example, the HM of mothers with male infants had 25% greater milk energy content than mothers delivering female infants (185). Furthermore, at 4 months of age, HM for male infants compared with females were higher in energy and lipid content by 24 and 39%, respectively

TABLE 3 | Infant-related factors affecting nutritive and non-nutritive components of human milk.

Factors	Effect on nutritive or non-nutritive components of HM	Evidence	References
Birth weight	Higher birth weight increases HM intake	Evidence probable	(174–176)
Gestational age at birth	Preterm birth increases true protein in colostrum	Evidence probable	(177–184)
	Preterm birth decreases lactose in colostrum	Evidence probable	
	Preterm birth decreases minerals, Ig, growth factors	Lacking	
Sex	Male infants consume higher amounts of HM than female infants	Evidence probable	(185–188)
	Energy and lipid content of HM is higher after giving birth to male infants	Inconsistent or lacking	
Age (stage of lactation)	Advanced stage of lactation increases HM intake, as well as energy, total lipids and HMO (3 FL) content	Evidence probable	(4, 7, 98, 137, 186, 189–213)
	Advanced stage of lactation has no major impact on lactose and some HMOs (e.g., 3 SL)	Inconsistent or lacking	
	Advanced stage of lactation decreases total and major proteins, as well as some HMOs (e.g., 2 FL), immune factors, whey/casein, vitamins and zinc	Evidence probable	
Feeding session	Hindmilk has higher energy and total lipids compared with foremilk	Evidence probable	(195, 196)
Feeding—time of the day	Mid-day feeding demonstrates higher energy and total lipids	Lacking	(214, 215)
Variations between breasts	Milk output from the right breast is greater than from the left breast	Inconsistent	(195, 196, 216–224)
	Energy content in HM from the left breast is higher than from the right breast	Inconsistent or lacking	
Circadian variability	Lipids and lipolytic enzymes in HM peak at mid-day	Evidence probable	(150, 214, 215, 225–239)

(186). The same study revealed lipids such as linoleic acid, phospholipids and gangliosides were increased in HM of male infants in later lactation, potentially based on the higher energy requirement of a male infant (186). Interestingly, in resource poor setting, HM for female infants was richer in lipids, while in a resource sufficient setting, the milk for male infants was richer suggesting that infant sex and maternal socioeconomic status showed an interaction and should be studied further (187). HM intake, assessed using stable isotope methodology, was shown to be higher in males than in females over the first 12 months of lactation, likely related to the fact that males have greater lean mass than females during infancy (188).

Temporal Changes in HM Volume and Composition

Changes in HM Volume Across Lactation

HM is a dynamic fluid that changes its composition and volume depending on the stage of lactation and adapts to the evolving nutritional requirements of the infant (4, 189–192). Stable isotope studies in infants aged 0–24 months have shown that the volume of HM intake increased over the first 3–4 months and remained above 0.80 kg/d until 6–7 months in order to meet the increasing energy requirements of the infant (188).

Branched-chain FAs may reduce the incidence of necrotizing enterocolitis, but evidence is limited. One study found branched-chain FAs decreased with increased lactation (193). Most growth factors (EGF, IGF, and TGF) significantly decrease over the course of lactation from colostrum, over transitional and mature milk, while TGF-α content in HM revealed a significantly increased trend over the course of lactation (137). The time of lactation had an influence on the concentration of oligosaccharides, on 4 out of 22 oligosaccharides measured. (194). However, published data are hampered by large variation and results need to be interpreted with caution.

Changes Observed Within Feed and Between Breasts

The energy content of HM is not constant and changes within a feeding (195). Significantly higher energy content has been observed in hind milk than in foremilk since the lipid content increases markedly with emptying of the breast. The fat content of hind milk is reported to being approximately two- to three-fold that of foremilk and included 25–35 kcal/100 mL more energy on average than foremilk (195). Milk volume differences between left and right breasts have also been a topic of study. Reports indicated that milk output from the right breast was usually greater than the left breast (216–219, 240). However, the opposite has also been reported where the left breast showed a greater milk output in pump-dependent mothers of non-nursing preterm infants (220). Perhaps studying physiological implications of left cradling bias that occurs in 70–85% of the women may help shed light on the observed between breast variability (221). When milk composition from the left and right breasts was assessed over 12 months in fore and hind milk the content of fat was found to vary slightly between breasts, with a mean CV of 47.6 (SE 2.1) % (*n* 76) and 46.7 (SE 1.7) % (*n* 76) for left and right breasts (196). These variations resulted in differences in the amount of fat delivered to the infant over 24 h. This indicated that a rigorous sampling routine should take into account all levels of variation in order to accurately determine infant HM intake (196). Different levels of sodium, chloride, glucose, lipid, and zinc were reported in

the HM of breasts but differences were either transient and/or random (222). In a different study the levels of sodium, the levels have shown a difference, potentially due to underlying mastitis localized to only one breast (223).

Changes Due to Circadian Variability

Diurnal variation in the lipid content of HM was first studied more than a century ago (225). Later it was shown that diurnal variations were unrelated to the degree of emptying of the breast in the previous feeding and to maternal meal times (226). Since then it has been a topic of interest for many teams of scientists in normal term (150, 227–229, 241) as well as preterm infants (230). It is noteworthy that not only lipids in HM but lipolytic enzymes of HM show a circadian rhythm and peak at mid-day, which has been shown for serum-stimulated lipase and lipoprotein lipase but not bile salt-stimulated lipase (214, 215). In a study with 6 women, HM volume, along with total lipids were found to be significantly higher between 08:00–12:00 and 16:00–20:00 (231). The same team suggested that if 24-h sampling was not possible, single samplings at 12h00, 20h00, and 24h00 may be most representative for lipid yield and may predict the value within 97–124%. Since lipids provide more than 50% of total energy in HM, it is not surprising that energy content of HM was positively related to fat content (232). Though lipids have been studied the most, milk minerals also have received some attention. However, the evidence was not very conclusive and did not demonstrate a cyclic change at a different stage of lactation (233–237). Endocrine factors and melatonin are hypothesized to improve nocturnal sleep and reduce infantile colic (238). Indeed, it has been shown that melatonin is found at significantly higher concentrations in night time HM (7.3 pg/mL) as opposed to day time HM (1.5 pg/mL) (239).

Longitudinal Changes Across Lactation

Among all the factors covered in this paper, longitudinal changes observed across progressing stages of lactation have been studied the most. Key differences include the presence of a relatively high abundance of immune related factors in early compared with later stages of lactation (7). Additionally, a higher concentration of total proteins has been observed in colostrum and transitional compared with mature milk (190). In contrast, lipids are present at higher concentration in mature milk (186, 196). In next section we review the different aspects of changes across lactation as reported in the literature.

Changes in the macronutrient composition of HM

The energy content of HM showed an initial decrease followed by an increase over the course of lactation, which was directly related to the HM lipid content (186, 196). With respect to carbohydrates, several studies showed that lactose concentrations remained constant throughout lactation (186, 195, 197), however, there is also some evidence to the contrary, indicating an increase in HM lactose from 56 to 69 g/l over the first 4 months of lactation (198). The other significant carbohydrates found in HM are oligosaccharides, the concentrations of most of which, not all, have been shown to decline during the course of lactation although HM concentrations of 3-FL either increased throughout lactation or remained at relatively constant concentrations starting at 1 month onwards (199, 200). The colostrum to milk transition was also associated with significant changes in concentrations of free sugars and polyols (increased in concentrations of lactose and glucose, decreased in concentrations of Myo-inositol and glycerol) over the first 3 days after birth and thereafter reaching a steady state (201).

Colostrum contains very high concentrations of protein (ranging from 20 to 30 g/L), which decreases significantly thereafter, and reaches 7–8 g/L after 6 months of breastfeeding (202). This observation coincides with lower protein intake and lower growth rate of breastfed infants in the latter half of the first year of lactation (203). A systematic review reported on the reduction in HM protein concentration (g/L) from about 25 in colostrum (age 1–5 days) to 17 in transitional milk (age 6–14 days) and then to 13 (8–21) in mature milk (at age of >14 days and <6 weeks) (204). The protein profile of the HM also evolves over lactation. At the beginning of lactation the whey: casein ratio was around 90:10, reduced to 70:30 in transition milk, then to 60:40 in mature milk and ultimately reached 50:50 ratio in mature milk after 6 months of lactation (205, 206). The concentration of lactoferrin in HM (second most abundant whey protein in HM known for its antimicrobial activities) was high during early lactation (<28 days lactation), decreased by almost 50% in the first 5 days of lactation, and thereafter remained relatively stable (207). The total nitrogen and total AAs in HM decreased in the first 2months of lactation and thereafter remained relatively unchanged, while the free AAs (glutamic acid and glutamine) increased over lactation, peaked around 3–6 months, and thereafter decreased (208).

The most variable macronutrient in the HM is fat, which is dependent on a number of factors and includes the fat content of colostrum; it is usually very low (1–2%) but was shown to increase rapidly during the first week of lactation (204). The mean lipid content of HM (g/L) increased from 22 in colostrum (age 1–5 days), to 30 in transitional milk (age 6–14 days), and 38 in mature milk (age >14 days and <6 weeks) (204). There were also stage-based changes reported in the ganglioside content of HM with some decreasing and others increasing over the time (209).

Changes in micronutrient composition of HM

The concentrations of antioxidant vitamins such as A and E are known to be lower in mature milk compared with colostrum and decrease over the course of lactation, while the total antioxidant status of mature milk was higher than colostrum indicating an increase in total antioxidant properties (210). Non-provitamin A carotenoids (lutein and lycopene) decreased while there was no significant change in the provitamin A carotenoids (α- and β-cryptoxanthin) with advancing lactation stage (211). Over the course of lactation (up to 240 days) significant changes of carotenoids and tocopherol were reported in a population of 540 Chinese women (98). In this population, the concentrations of lutein, zeaxanthin, and α-tocopherol were higher the first 4 days, decreased until 12 days post-partum to remain stable afterwards. Zeaxanthin and γ-tocopherol remained stable over time.

Zinc concentrations in HM decline dramatically over the first 3–5 months of lactation and even an increase in HM volume during the early weeks post-partum is not sufficient to overcome this decline (212). A marked decrease in HM

TABLE 4 | Methodological factors affecting nutritive and non-nutritive components of human milk.

Factors	Effect on nutritive or non-nutritive components of HM	Evidence	References
Human milk expression	Hand expression compared with pump expression shows lower daily milk supply	Inconsistent or lacking	(242–247)
	Hand expression compared with pump expression results in higher sodium, potassium, proteins, total lipids	Inconsistent	
Storage temperature	Higher temperatures (−20°C) reduce total lipids	Inconsistent or lacking	(248–255)
Storage length	Longer storage durations reduce vitamin C concentrations	Inconsistent or lacking	(248, 252–255)
	Longer storage durations do not affect tocopherol concentrations	Evidence probable	
	Longer storage durations (12 months) reduce concentrations of IgA, IL-8, and TGF-β1	Inconsistent or lacking	
Freeze-thaw cycles	Multiple cycles reduce total lipids	Evidence probable	(250, 251, 256, 257)
	Multiple cycles reduce carbohydrate concentrations	Lacking	
	Multiple cycles increase lipolytic products (free FA, monoacylglycerol)	Evidence probable	
Choice of analytical method	Determination of combustion in a bomb calorimeter accurately quantifies macronutrients and total energy	Evidence probable	(177, 205, 258–273)
	The Kjeldahl method or micro-Kjeldahl analysis measure true protein with high precision	Evidence probable	
	High throughput spectroscopy measures protein accurately	Inconsistent	
	The Folch, Bligh and Dyer and Röse-Gottlieb methods accurately measure lipid content	Evidence probable	
	Spectroscopic methods determine total lipids accurately	Inconsistent	

cobalamin was also observed at 4 months and this has been associated with an accompanying decrease in plasma cobalamin and holotranscobalamin in infants, indicating an impaired cobalamin status (213).

Methodological Factors Affecting the Quantification of HM Components in a Research Setting

The main factors identified have been summarized in **Table 4**.

Sample Collection—Hand Expression vs. Electric Pump Expression

It is well-known that there are multiple ways of expressing milk from mothers' breasts, be it for feeding to the new-born, for donation or for research purposes. The methods may include but are not limited to, hand expression, manual pump and electric pump (242). In a randomized trial of mothers delivering very low birth weight infants, hand expression produced significantly lower cumulative daily milk volume in comparison with electric pump expression (243). Sodium concentration was shown to be significantly affected by method of pumping. Higher sodium content was found in hand expressed milk as opposed to electric pump-expressed milk (244). In a Cochrane review of methods for HM expression, sodium, potassium, protein, and fat constituents of HM were reported to differ between methods. However, no consistent effect was found related to prolactin change or effect on oxytocin release with pump type or method (242). A randomized trial indicated that HM expressed manually contained higher fat than that expressed electrically (245). Morton et al. (246) indicated that a combination of manual technique and electric pumping resulted in high levels of fat-rich, calorie-dense milk, unrelated to production differences. An experiment from 57 lactating women showed that macronutrient content (fat, carbohydrate, protein, and energy contents) of mid expression HM is unaffected by maternal handedness, breast size or breast side dominance (247, 274).

Post-collection Handling of HM Samples

Utmost care must be observed with HM samples immediately after the milk is expressed from the mothers' breast into the collection container. Collection tubes may need to be acid washed prior to collection if the intention is to measure minerals. Similarly, maintenance of a cold chain until laboratory analyses, ensuring optimal storage temperature and duration, limiting freeze-thaw cycles, amount of oxygen exposures etc. comes into the scope of post-collection handling variability. It is our general observation that not many systematic studies exist that assess and guide the future HM research scientist, but few observational as well as data supported statements are mentioned in this section.

Storage temper and duration

Lipids, more than carbohydrates or proteins in HM, are sensitive to storage over long periods. Chang and colleagues demonstrated that even storage at −20°C for 2 days leads to ∼9% reduction in HM total lipid content (248). It is likely that bile salt-stimulated lipases present in HM may still maintain some activity at cooler temperatures and lead to enzymatic hydrolysis of lipids (249). Indeed this was confirmed in a separate study where storage at −20°C was also done with pre-heating milk samples at 80°C (90 sec) to destabilize the lipolytic enzyme (250). Therefore, if possible, samples should be stored at the minimum possible temperature, preferably at −80°C, from the day of collection. However, others have argued that freezing at −80°C reduced the energy content of both fat and carbohydrates and suggested HM should be stored at −20°C (251). Furthermore, even vitamin C was reduced significantly over time in cold storage but not tocopherols and total FAs (252, 253). Bacterial composition (quantitative or qualitative) of HM was stable with cold storage at −20°C for 6 weeks (254). Long-term storage of HM may affect immunological composition. According to Ramirez-Santana et al. freezing storage of colostrum at −20 and −80°C for a 12-month period produced a decrease in the concentrations of IgA, IL-8, and TGF-β1 (255).

Freeze-thaw cycles

Freeze (−20°C) and thaw (5°C) cycles have been shown to activate lipolysis and increase the presence of lipolytic product in HM, such as free FAs, monoacylglycerols and diacylglycerols (250, 256). Structural effects such as increasing size of milk fat globules due to freezing have also been observed (257). Carbohydrate content was significantly reduced in thawed HM samples when compared with fresh samples (251).

Analytical Procedures

The choice of analytical methods to measure the nutrient of interest in HM is important, especially in comparative and longitudinal studies, where differences among populations and lactation stages could be due to biological variation or may reflect methodological biases and errors. Several factors influence the analysis of HM and in the following paragraph, we will discuss the most relevant ones. This is not intended to be a review on methodologies for determination of nutrients in HM, but to demonstrate the consequence of using unsuitable methodologies.

Sample preparation

Using the correct preparation and storage of samples are important elements of maintaining the integrity of HM during storage, as previously mentioned. In our experience, maintaining sample homogeneity is an issue both when frozen samples are thawed at room temperature or in a 37°C water bath. Often, probe sonication should be used if the measure is based on optical analyses, such as the commonly used mid infrared spectroscopy. HM must be immersed in warm water (30–40°C) and gently mixed, prior to analysis, in order to rapidly thaw and minimize phase separation. Homogenization is an important step in preparing milk samples for total fat determination by infrared (IR) spectroscopy techniques, as it decreases the variability in fat globule size and the light-scattering effect of larger globules, improving the accuracy of the measurement (258).

Selectivity/Specificity and accuracy

When quantitative data are required, the analytical methods chosen must demonstrate enough selectivity and accuracy. Selectivity is the ability of the method to distinguish between the analyte and other substances present in the matrix. In absence of selectivity, accurate values cannot be obtained. The accuracy expresses the closeness of a result to an accepted reference value and it is normally studied as two components: trueness, which represents the closeness of agreement between the average value obtained from a series of results and a reference value and precision, which is the closeness of agreement between independent test results obtained under stipulated conditions (ISO 5725) (259). In the following paragraphs, several methodologies are discussed with respect to selectivity and accuracy.

Energy

Two approaches have been used to measure energy in HM: direct energy quantification by combusting in a bomb calorimeter and calculated energy using Nichols et al. (260) energy multiplication factors for protein, fat and carbohydrate. Lack of accuracy has been shown by Gidrewicz et al. (177) for the measurement of energy when calculated using values for the energy contributions from fat, protein and carbohydrate rather when measured by bomb calorimetry. Differences as high as 13%, which likely represent clinically important differences, were reported (177). Since HM contains a large amount (∼12%) of non-digestible oligosaccharides, they will contribute a significant amount of energy when using bomb calorimetry for energy measurement, but since they are essentially non-digestible, they should contribute significantly less energy to the infant. Overall, the choice of analytical methods can determine outcome and interpretation of HM analyses. Therefore, adoption of standard methods when possible and/or rigorous assessment of alternative methods is needed. To assure equivalence of two analytical methods, we recommend evaluating the accuracy of the alternative method and to check the presence of systematic and/or proportional bias by performing analysis of differences and a regression analysis, respectively.

TABLE 5 | Recommendations for collecting and handling of HM samples.

Collection of samples
Standardize the time of sampling of HM to avoid diurnal variations
Agree on breasts for HM collection
Request mothers to completely empty the breast, ∼2 h prior to collection of milk by feeding the infant or manual pumping to minimize the impact on nutrient concentration of interest by residual milk from previous feeding session
Realize single full HM sampling to ensure inclusion of fore-, mid-, and hind milk
Record the volume of milk
Standardize the collection of milk for all study subjects by either electrical pumping or hand pumping to minimize variation due to extraction method
Handling of samples
Store milk in smaller aliquots (e.g., 250 μL/aliquot) to avoid freeze-thaw cycles if multiple analyses are anticipated
Store aliquots of milk in tubes at −80°C as quickly as possible and at temperatures as low as possible in field collections until transfer to central laboratory for longer term storage
Analyses
Choose appropriate analytical method to characterize and quantify nutrients in HM, with respect to their selectivity, accuracy and precision

Proteins

Amino acid analysis is the most accurate method for determining true protein content (205). The Kjeldahl method, based on complete combustion of the sample, measures total nitrogen (TN) and it is the most accurate indirect determination of total protein content in HM (261). The true protein content can also be estimated by the chemical determination of non-protein nitrogen (NPN) (262), and subsequent subtraction from TN. Unfortunately, AA analysis is costly and time consuming and the Kjeldahl method requires large amounts of sample. Alternatively micro-Kjeldahl (263) analyses can be used with high precision to determine true protein because it has been shown to correlate well with AA analysis (264) if corrections are made for NPN. High throughput approaches requiring low sample amounts such as spectroscopy and colorimetric methods have been investigated (258, 262, 265–268). Absence of accuracy was shown (258, 266) when comparing near- and mid IR spectroscopy equipment for determination of total protein against chemical reference methods. Casadio et al. (265) compared Miris HMA vs. Bradford

for total protein determination and reported overestimation of protein content. On the other hand, Silvestre et al. (267) reported underestimation of protein content when Miris HM analysis was compared vs. Bradford for total protein determination. This observation illustrates that spectroscopy methods can vary in accuracy depending on the calibration of the instrument and reference method; hence the appropriate methods should be selected with care. Keller and Neville (262) compared micro-Kjeldahl to different spectrophotometric methods to determine proteins in HM: Biuret assay, Lowry-Peterson assay, Bio-Rad Coomassie Blue assay and Pierce Bicinchoninic acid assay (BCA). The Pierce BCA assay demonstrated better accuracy, and was recommended for total protein analysis in HM. Lonnerdal et al. (268) showed the BCA method consistently overestimated Kjeldahl protein by 30%. However, parameters such as incubation time and temperature highly influenced the accuracy of the BCA method (269).

Lipid

In order to accurately quantify HM lipid, the first step is disruption of the biological membrane enveloping triacylglycerol core, e.g., ultrasound treatment, followed by lipid separation from milk protein by the addition of organic solvent [e.g., methanol (270, 271)] or acids or bases [NH_3 (272)] which triggers protein precipitation. At this point, lipid can be extracted from the matrix by using organic solvents. Since most of the lipid in HM is represented by triacylglycerol (98%), solvents like diethyl ether and petroleum benzene (272) can be used. Lipid extraction methods are time consuming and other approaches like Gerber, creamatocrit, and spectroscopy have been applied for the quantification of lipid in HM. The Gerber method is based on protein precipitation and further separation of fat by centrifugation. A collaborative study (273) performed on crude and homogenized pasteurized cow milk showed that the Gerber method underestimated the amount of fat by 0.02–0.06% compared with the official method of Röse-Gottlieb. A critical point of the method is the accuracy of butyrometers. The supplier should calibrate the butyrometer at delivery and supply a test certificate. The internal surface of the butyrometers must be smooth and free of defects. Recently, spectroscopy techniques have been employed to measure total lipid in HM, and showed conflicting results on precision and accuracy of these techniques (258, 265, 266). The reasons were mainly due to selection of chemical reference methods for the comparison and calibration of spectroscopy instruments. Therefore, the Folch, Bligh, and Dyer and Röse-Gottlieb extraction methods are highly recommended to perform absolute quantification of lipid in human milk (261) and they have to be used for calibrating near- and mid IR spectroscopy equipment.

Lactose

Indirect methods, such as colorimetric methods can show poor selectivity. Casadio et al. (265) reported the lack of selectivity of the enzymatic essay to measure lactose in HM, due to lactose overestimation when compared with HPLC measurements. Attempts have been made to quantify lactose by near- and mid IR spectroscopy (258), however neither of the approaches were accurate or precise (261, 266).

Below the main considerations, for successful HM collections and analyses in observational as well as interventional studies, are summarized (see **Table 5**).

CONCLUSIONS AND FURTHER RECOMMENDATIONS

To our knowledge, this is the first comprehensive literature review that provides a holistic overview of a broad range of factors affecting HM composition and volume. Several maternal and infant characteristics as well as study methodological aspects were found to impact HM composition and volume. Study procedures should be systematically recorded and/or standardized in individual studies, whenever possible, to facilitate appropriate analysis, and interpretation of data and comparisons across studies. HM lipids and lipophiles were the nutrients found to be most vulnerable to maternal, infant and methodological factors. We therefore recommend standardizing the sampling of the HM, especially for characterization of lipids, lipophiles, and for those nutrients whose variability is yet unknown. Keen attention should be given to the choice of the analytical method to characterize and quantify nutrients in HM, with respect to their selectivity, accuracy and precision.

Our review not only summarizes recommendations for standardization of HM sampling and handling for future research protocols, but also sheds light on the dynamic and highly variable nature of HM. This knowledge is crucial to better understand the controversial impact HM may have on developmental outcomes of infant early life. We also illustrate the agility of HM to adapt to changes in the environment of the mother and evolving nutritional requirements of the infant. There is an apparent need of systematic reviews of existing evidence in the field. It is particularly crucial to better understand those maternal dietary, lifestyle, and environmental exposures that can be modified to significantly impact HM composition and thereby positively impact infants' developmental and health outcomes.

AUTHOR CONTRIBUTIONS

ST conceived the idea of the review paper. TS, QZ, FG, and ST wrote sections of the manuscript with critical inputs from DM and VV. All authors read and approved the final version of the manuscript.

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Degradation of Proteins From Colostrum and Mature Milk From Chinese Mothers Using an *in vitro* Infant Digestion Model

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This study provided insights into the degradation of human milk proteins in an *in vitro* infant digestion model by comparing colostrum (week 1) and mature milk (week 4) of 7 Chinese mothers individually. In this study, we adapted the exiting INFOGEST *in vitro* model, to conditions representative to infants (0 to 3 month-old). The level of undigested proteins was analyzed by LC-MS/MS after gel-electrophoretic separation and in-gel digestion. The BCA protein assay showed that the total undigested milk protein content decreased from the start to the end of digestion with variations between mothers, especially in the gastric phase (25–80%). Undigested proteins could also still be found after the intestinal phase, ranging from 0.5 to 4.2% of initial protein content. Based on LC-MS/MS analysis, milk protein digestion varied between the mothers individually, especially during the gastric phase. No differences could be observed between protein digestion from colostrum and mature milk after the intestinal phase. The highest levels of proteins remaining after intestinal digestion can be linked to the group immune-active proteins, for all mothers. The level of protease inhibitors and total protein content in the milk did not correlate with the overall proteolysis during digestion. The results also showed that milk serum proteins partly remained after the gastric phase, with 33% remaining from colostrum and 37% remaining from mature milk. More than 40 milk serum proteins were detected after the intestinal phase. Some of the highly abundant milk serum proteins (lactoferrin, serum albumin, bile salt-activated lipase, immunoglobulins, α_1-antichymotrypsin) were still partially present intact after the intestinal phase, for all mothers. Caseins were also not completely digested in the gastric phase, with 35% remaining from colostrum and 13% remaining from mature milk. Caseins, on the other hand, were almost completely digested after the intestinal phase. The complete degradation of caseins into peptides might be related to their structural features. Overall, this study showed that digestion differed for the various human milk proteins by adapting an *in vitro* digestion model to infant physiological conditions, with the main differences between digestion of the milk from individual mothers being observed after gastric digestion.

Keywords: human milk proteins, protease inhibitors, casein, serum proteins, milk protein digestion

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INTRODUCTION

Human milk is a complex mixture of nutrients and bioactive constituents, contributing to the infant's growth, development and health (1). Human milk proteins, amongst others, play a pivotal role in protecting the infant's gut mucosa against pathogens (1). There are two distinct types of proteins in human milk, caseins, and serum proteins, with changing quantities and ratios over lactation (1). Caseins (α_{S1}, β-, and κ-casein) are generally described as transport proteins due to their calcium-binding properties, and become bioavailable after digestion (2). The hydrophobic regions of caseins consist of a high number of proline residues, which prevents the formation of close-packed secondary structures (3). The serum proteins in human milk have many different functions (4). The most abundant serum protein groups in human milk are enzymes (e.g., α-lactalbumin, bile salt-activated lipase), transport proteins (e.g., serum albumin, fatty acid-binding protein), and immune-active proteins (e.g., lactoferrin, immunoglobulins) (5). This latter study also showed that the milk serum protein composition varied among mothers and between different populations (5).

It has been assumed that caseins in human milk are fully digested in the infant's digestive tract, facilitating the uptake of relatively small peptides, essential amino acids and minerals associated with the micelles (6–8). Human milk serum proteins that do not have an extensive tertiary structure, e.g., polymeric immunoglobulin receptor (PIGR) and osteopontin, also may be broken down completely during infant digestion, in contrast to more tightly folded milk serum proteins like lactoferrin and immunoglobulins (9–13). Some of the major proteins (caseins, osteopontin, clusterin, PIGR) may be predigested by proteases, resulting in the presence of peptides in undigested human milk (12). A variety of studies have reported that specific serum proteins like lactoferrin, immunoglobulins, and α_1-antitrypsin from human milk can be found intact in the stool of breastfed infants, showing that those proteins are able to partially survive digestion in the infant's digestive tract (14–18).

It has been suggested that the extent of protein digestion might be reduced by the presence of protease inhibitors (e.g., α_1-antichymotrypsin and α_1-antitrypsin) or by the high total protein levels in human milk. Protease inhibitors might inhibit the function of trypsin and other serine proteases during small intestinal digestion (19–22). Colostrum contains a relatively higher quantity of protease inhibitors than mature milk (5), which might lead to more undigested proteins from colostrum at the end of digestion. In addition, protein digestion might also be influenced by the total protein content in colostrum. Colostrum contains a higher total protein content (14–16 g/L) compared to mature milk (7–10 g/L) (1). Therefore, it would be of interest to investigate both the variation in level of protease inhibitors, as well as total protein content, in relation to the degree of protein hydrolysis.

Different static *in vitro* digestion models have been developed over the years, mimicking the gastrointestinal tract of adults and 3 year-old infants (23–31). Although static *in vitro* digestion only partly represents the digestive tract, the INFOGEST adult *in vitro* model has been shown to mimic *in vivo* digestion of bovine milk proteins (32, 33). Colostrum (between 0 and 2 weeks) and mature milk (>4 weeks postpartum) are quite different in protein content and composition (4). Based on the gene expression of the infant's gastrointestinal tract, infants at 4 weeks of age have amongst others 40% of their chymotrypsin capacity, and only 10% of their pepsin capacity available compared to adults (34). To elaborate and better comprehend the digestion of proteins from colostrum and mature milk, the preexisting INFOGEST *in vitro* digestion model was adapted representing 0 to 3 month-old infant's digestion. This model is different from the existing adult *in vitro* digestion model (23), by having a higher gastric pH of 5, lower enzyme activities (pepsin 200 U/mL in gastric phase; trypsin 8.33 U/mL in intestinal phase), and shorter transition times (1 h each for gastric and intestinal phases) (34). Based on these modifications, this would further mimic the situation in infants, who have generally decreased protein digestion than adults.

The aim of this study was to better understand the variation in protein digestion. The enzymatic hydrolysis of proteins in milk from 7 Chinese mothers from 2 different lactation periods (colostrum, week 1; mature milk, week 4) was investigated in an adapted *in vitro* digestion model representing 0 to 3 month-old infants. The level of undigested proteins was analyzed by a combination of bicinchoninic acid (BCA) protein assay, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and liquid chromatography–tandem mass spectrometry (LC–MS/MS).

MATERIALS AND METHODS

Sample Collection

Human milk was collected as described previously (5) and samples from 7 different Chinese mothers from 2 different stages of lactation (colostrum, week 1; mature milk, week 4) were used. The samples used in this study were aliquots, but not the same samples, as described previously (5). Healthy Chinese women who delivered singleton term infants (38–42 weeks) were eligible for this study. Human milk collection was approved by the Chinese Ethics Committee of Registering Clinical Trials (ChiECRCT-20150017). Written informed consent was obtained from these 7 mothers.

The Infant *in vitro* Protein Digestion Model

The INFOGEST static adult *in vitro* digestion model, as described previously (23), was modified to an *in vitro* infant (0–3 months) protein digestion model (34). In comparison to adults, the pH and porcine pepsin concentrations in the gastric phase were adjusted, as well as the porcine pancreatin and porcine bile salt concentrations in the intestinal phase. The time to mimic each digestion phase was changed to 1 h. Briefly, the milk fat was

Abbreviations: ABC, Ammonium bicarbonate; BCA, bicinchoninic acid; CaCl2, calcium chloride; DTT, dithiothreitol; FDR, false discovery rate; iBAQ, intensity based absolute quantification; IAA, iodoacetamide; LC–MS/MS, liquid chromatography–tandem mass spectrometry; LDS, lithium dodecyl sulfate; PIGR, polymeric immunoglobulin receptor; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

removed by centrifugation (10 min, 1,500 g, 4°C). Next, skim milk (8 mL) was mixed with 6 mL of simulated gastric fluid, after which 5 μL of 0.3 M calcium chloride ($CaCl_2$) and 695 μL of water was added. Porcine pepsin diluted in simulated gastric fluid was added to reach an enzyme activity of 200 U/mL instead of 2,000 U/mL for the adult model in the final gastric mixture. The pH of the chyme was adjusted to 5 instead of 3 with 1 M HCl. The mixture was then incubated at 37°C for 1 h while mildly shaking at 200 rpm. After incubation, the pH was adjusted to 7 with 1 M sodium hydroxide solution. For duodenal digestion, 7.5 mL of gastric chyme was mixed with 4 mL of simulated intestinal fluid electrolyte stock solution (23). Porcine pancreatin was added to reach a trypsin enzyme activity of 8.33 U/mL instead of 100 U/mL for the adult model. After that, 2.5 mL of bile salts (40 mM), 40 μL of 0.3 M $CaCl_2$, and 1.31 mL water was added. The pH of the chyme was then again adjusted to 7 with 1 M hydrogen chloride solution. The duodenal chyme was then incubated at 37°C for 1 h in a water bath while mildly shaking at 200 rpm. After incubation, the porcine pancreatin was inactivated with 50 μL of the irreversible serine protease inhibitor 4-(2-aminoethyl) benzenesulfonylfluoride (100 mM) in the duodenal chyme. The skim milk was diluted 4 times, while the samples after the gastric phase were diluted twice, facilitating direct comparison with the samples of the duodenal phase (34).

Total Concentrations of Proteins Before and After *in vitro* Digestion

The total protein concentration of the blank and digesta were measured in duplicate using the BCA protein assay kit 23225 (Thermo Scientific Pierce, Massachusetts, U.S.). Standards and reagents were prepared according to the manufacturer's instructions. Before analysis, 1 mL of the sample was mixed 1:1 with absolute trichloroacetic acid (TCA). After centrifugation (1,500 g for 30 min, 4°C), the supernatant containing peptides was removed. TCA-precipitated proteins were washed twice with cold acetone to completely remove TCA, and the pellet dried at 70°C in a heating block (Labtherm Graphit, Liebisch, DE) for 60 min. The dried proteins were re-dissolved in 2 mL of the BCA working reagent, and incubated at 37°C in a water bath for 30 min. After cooling down to room temperature, the samples were ready for spectrophotometric measurements. Based on the BCA calibration curve using bovine serum albumin, concentrations were expressed as g/L for the diluted samples.

SDS-PAGE, In-gel Digestion, and Purification by Solid Phase Extraction

For every sample, 2 μL was taken and diluted in 5 μL lithium dodecyl sulfate (LDS) sample buffer (pH 8.4, Life Technologies, Carlsbad, U.S.) and 15 μL water. This mixture was centrifuged at 1,500 g for 1 min and the supernatant heated at 70°C in a heating block for 10 min. Samples and pre-stained marker (5–165 kDa, Jena Bioscience, De) were then loaded onto NuPAGE 12% Bis-Tris gels (Life Technologies, Carlsbad, U.S.). Gels were run with an LDS running buffer (containing 50 mM MES, 50 mM Tris base, 0.1% SDS, 1 mM EDTA, pH 7.3) under non-reducing conditions at 120 V in a vertical electrophoresis cell (Bio-Rad, Hercules, U.S.). The gels were stained with colloidal Coomassie G-250 (Bio-Rad, Hercules, CA), followed by destaining with water and washing buffer (10% ethanol, 7.5% acetic acid in water). The gels were scanned after staining with Image Lab version 4.1 (Bio-Rad) to visualize the protein patterns and determine the location of α-lactalbumin on the gel.

In-gel digestion was used to digest proteins into peptides. As described previously (35), SDS-PAGE gels were incubated in 25 mL of ammonium bicarbonate (ABC) containing 0.039 g dithiothreitol (= 10 mM DTT, pH 8) for 1 h at 60°C. Subsequently, the gels were incubated in 25 mL of Tris buffer pH 8 containing 0.092 g iodoacetamide (= 20 mM IAA, pH 8) for 1 h at room temperature in the dark. To separate proteins (> 10 kDa) from small peptides, single lanes of the SDS-PAGE gels were cut into <1 mm^3 small pieces and pieces above the band of α-lactalbumin were transferred to 1.5 mL Eppendorf low protein binding tubes. The gel pieces were frozen and thawed 3 times to increase the accessibility for trypsin. Then, 100 μL of 50 mM ABC (pH 8) containing 0.5 μg trypsin was added to the gel pieces, followed by 100 μL ABC to cover the gel pieces completely. According to the manufactures, the activity of bovine sequencing grade trypsin was ≥7,500 benzoyl-L-arginine-ethyl-ester U/mL protein (Roche, Basel, CH). After trypsin digestion overnight, 15 μL of 10% trifluoroacetic acid in water was added to adjust the pH between 2 and 4 (pH-indicator strips).

Solid phase extraction was done to purify peptides, as described previously (12). Stage tips containing 2 mg Lichroprep C18 (25 um particles) column material (C18+ Stage tip) were made in-house. The peptides were transferred to a methanol washed and 0.1% formic acid equilibrated C18 stage tip column. The peptides were eluted with 50 μL of 50% acetonitrile in water containing 0.1% formic acid. The samples were dried in a vacuum concentrator (Eppendorf, Nijmegen, NL) at 45°C for 1 h until the volume of each sample decreased to 15 μL or less. The content of the tubes was transferred to empty low protein binding tubes, and samples reconstituted to 50 μL by adding water containing 0.1% formic acid.

LC-MS/MS and Data Analysis

LC-MS/MS was used to measure the amounts of distinct peptides. As described previously (35), 18 μL of each sample was injected on a 0.10 × 30 mm ProntoSil 300-3-C18H (Bischoff, Leonberg, DE) pre-concentration column (prepared in house at a maximum of 270 bar), peptides were eluted from the pre-concentration column onto a 0.10 × 200 mm ProntoSil 300-5-C18H analytical column, and the full scan FTMS spectra were measured in positive mode between m/z 380 and 1,400 on a LTQ-Orbitrap XL (Thermo Fisher Scientific, Waltham, U.S.). MS/MS scans of the four most abundant doubly- and triply-charged CID fragmented peaks in the FTMS scan were obtained in data-dependent mode in the linear trap (MS/MS threshold = 5.000) (35).

MS/MS spectra for each run were obtained and analyzed using the built-in Andromeda search engine with the Uniprot human protein database (36). Protein identification and quantification

was done as described previously (4). Maxquant created a decoy database consisting of reversed sequences to calculate the false discovery rate (FDR). The FDR was set to 0.01 on peptide and protein level. The minimum required peptide length was seven amino acids, and proteins were identified based on a minimum of two distinct peptides. The intensity based absolute quantification (iBAQ) values were used, representing the total peak intensity as determined by Maxquant for each protein, after correction for the number of measurable peptides (5). The iBAQ values have been reported to have a good correlation with known absolute protein amounts over at least four orders of magnitude (37).

The Uniprot database was also used to assign functions to all individual identified proteins, as described previously (5). The iBAQ values for each protein were used individually and summed per function, and per digestion phase (predigestion, gastric phase, intestinal phase). The iBAQ values of the proteins individually and grouped per function per phase were also converted in percentages of the total iBAQ intensity. The total iBAQ intensities of the skim milk from colostrum and mature milk were set to 100%.

To compare colostrum and mature milk from 7 Chinese mothers on total protein (based on the BCA protein assay) after both gastric and intestinal digestion, a *t*-test for independent samples was used (R, Lucent Technologies, New York, U.S.). For this comparison, the total BCA protein concentrations were preferred over the summed iBAQ values. For statistical analysis, an FDR adjusted $p < 0.05$ was considered significant. Scatterplots were generated with both the total protein (based on the BCA protein assay) and the levels of protease inhibitors (iBAQ values) in human milk against the relative change of total protein from milk to gastric and to intestinal digestion.

RESULTS AND DISCUSSION

Determination of Total Protein Before and After Digestion by the BCA Protein Assay

The existing INFOGEST *in vitro* digestion model was adapted to represent 0 to 3 month-old infant's digestion. The parameters of this adapted *in vitro* model, which was based on literature (34) including references and citations in that paper, represents the *in vivo* infant digestive conditions better than the adult model. Bovine milk serum was used for method development and validation of the model, as more was known about *in vitro* digestion of bovine milk proteins in older infants (2, 6, 11). As the findings for bovine milk were showing similar trends with previous studies (2, 6, 11), although using different age-specific models, the 0 to 3 month-old infant *in vitro* digestion model was then used for human milk samples.

The disappearance of human milk proteins was quantified using the BCA protein assay. Information was obtained on the total protein content before and after digestion of milk from 7 mothers and from 2 lactation periods (colostrum, week 1 and mature milk, week 4). The total BCA protein concentrations in skim milk ranged from 7.1 to 16.6 g/L for colostrum, and from 6.6 to 13.8 g/L for mature milk (**Figure 1A**). The lowest protein concentrations in colostrum (7.1 g/L) and mature milk (6.6 g/L) were from mother 7, whereas the highest protein concentrations in colostrum (16.6 g/L) and mature milk (15.3 g/L) were found from mothers 4 and 1, respectively (**Figure 1A**). The average BCA protein concentrations for colostrum and mature milk were 12.2 and 10.7 g/L, respectively. Colostrum contained higher total protein concentrations compared to mature milk, although the rate of decline varied among mothers during digestion (**Figure 1A**).

It can be observed that the total BCA milk protein content decreased from the start to the end of digestion with a large variation in the decline between mothers in the gastric phase (25–80%) (**Figure 1B**). The total BCA protein concentrations in colostrum for both mother 5 and 3 started at 13.3 and 11.7 g/L (**Figure 1A**), although showing the lowest (19.6%) and highest (75.3%) decline during gastric infant digestion of milk proteins (**Figure 1B**), respectively. It was also observed that still some undigested proteins could be found after intestinal digestion, ranging from 0.5 to 4.2% of total protein content (**Figure 1B**). The higher starting total protein concentrations in colostrum did not seem to be associated with a higher degree of total undigested proteins, as will be discussed in more detail later.

The Most Abundant Proteins in Human Milk as Identified by SDS-PAGE

The undigested proteins from human milk samples were initially monitored using an SDS-PAGE before performing in-gel digestion and the LC-MS/MS analysis. An example of an SDS-PAGE gel is given in **Figure 2**, showing the outcomes of *in vitro* digestion of the most abundant proteins in colostrum and mature milk from a single mother. It can be seen from **Figure 2** that caseins and α-lactalbumin from colostrum and mature milk were not readily digested in the gastric phase, but were completely digested after intestinal phase. Lactoferrin and serum albumin, on the other hand, were still partially present after the intestinal phase (**Figure 2**). Bands of the individual human milk proteins on the SDS-PAGE gels were not quantified, however, it can be observed that proteins in mature milk were digested to a rather similar extent by the *in vitro* infant digestion model as colostrum (**Figure 2**). The undigested human caseins in the gastric phase, as shown in **Figure 2**, has not been reported before. However, this might be related to the casein micelle size in human milk, as it has been reported that the mean casein micelle size varies between mammal species (e.g., human, bovine, equine species) and that casein micelles in human milk are much smaller in size than bovine milk (24). It has also been previously reported that smaller casein micelles form a more compact and hence firmer gel network than larger casein micelles (38). The milk from bovine and human showed different degradation patterns when digested with adult gastrointestinal enzymes for 30 min at their respective pH values (pH 2.5 in gastric phase, pH 8.0 in the intestinal phase) (24). During adult gastric digestion, the caseins in human and bovine milk were poorly degraded with 69 and 39% of remaining protein, respectively (24). Further digestion of caseins with intestinal enzymes resulted in an extremely fast

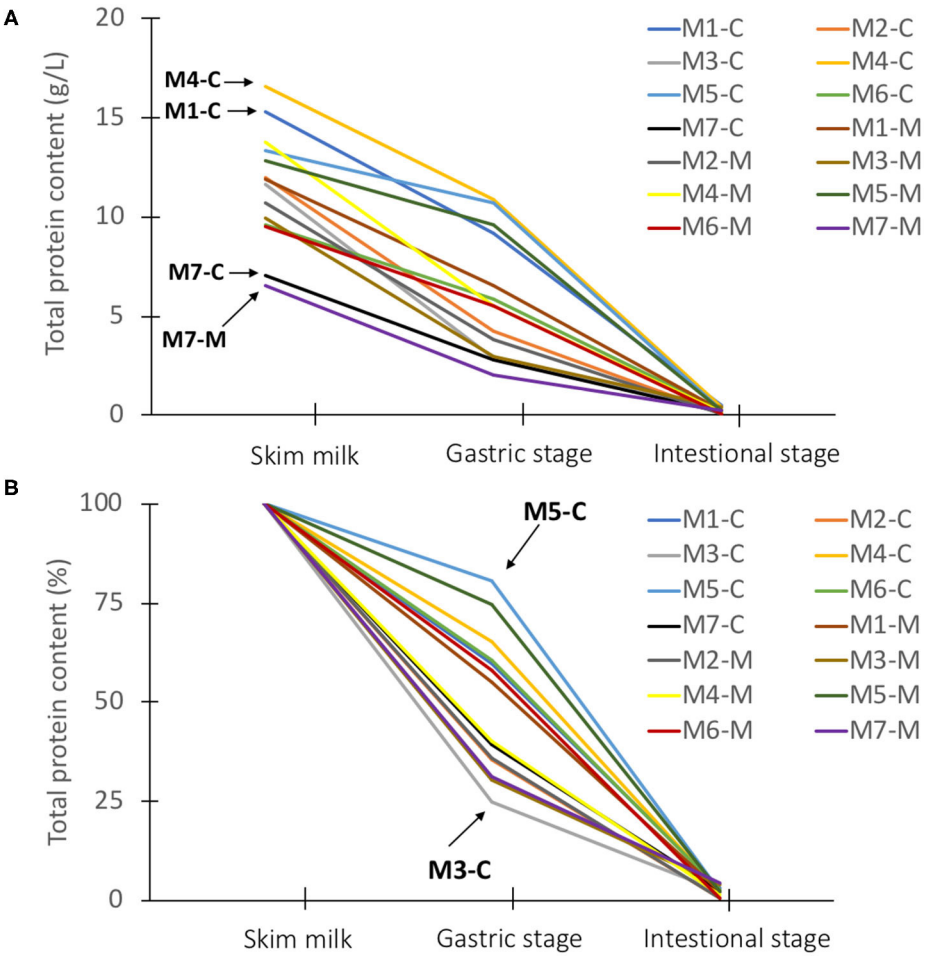

FIGURE 1 | The total BCA protein content (g/L) **(A)** and total BCA protein content (%) **(B)** in skim milk for colostrum (week 1) and mature milk (week 4) from 7 Chinese mothers after dilution of the samples, and after gastric and intestinal stage. The total BCA protein concentrations (g/L) in diluted skim milk for colostrum and mature milk of the individual mothers was set to 100%. MX; X = mother. The letter behind the hyphen indicates colostrum (C) and mature milk (M).

digestion of the caseins from all species (24), with 20% of the caseins remaining intact after 5 min, while after 30 min almost no intact caseins were left (24).

A randomized controlled trial with 12 hospitalized tube-fed preterm infants, showed that α-lactalbumin, lactoferrin, β-casein, and serum albumin were *in vivo* only partially digested in the infant's stomach (17), which matched with our findings (**Figure 2**). Overall, specific human milk serum proteins partially survived *in vitro* digestion, which will be further discussed while evaluating the findings obtained by LC-MS/MS.

LC-MS/MS Evaluation of the Protein Composition Prior to Digestion

Although a more quantitative analysis of the decrease in protein content was done by the BCA protein assay, this assay only determined the total protein content (**Figure 1**). A general decrease of individual proteins was observed by SDS-PAGE, whereas SDS-PAGE was only able to identify some of the most abundant human milk proteins (**Figure 2**). LC-MS/MS provided a more complete overview of the protein composition after the *in vitro* digestion phases. Lanes of the SDS-PAGE gels were cut above the band of α-lactalbumin, which was thus used as threshold for in-gel digestion, meaning that proteinaceous material >10 KDa was assumed to be identified and quantified as intact protein.

The average relative protein composition in human milk from 7 Chinese mothers from 2 different lactation periods can be found in **Figure 3**. Proteins present in both colostrum and mature milk were dominated by immune-active proteins, enzymes, and transport proteins (**Figure 3**). Colostrum contained relatively higher quantities of protease inhibitors, cell proteins, and "other," as compared to mature milk (**Figure 3**, insert). The relative levels of the latter protein groups were much lower than the transport, enzymes, and immune active proteins.

The higher relative levels of transport proteins in colostrum and mature milk in **Figure 3**, as compared to a previous study of the Chinese human serum proteome (5), might be explained by the presence of both free soluble and micellar caseins, whereas in the previous study during isolation of milk serum proteins, micellar caseins were completely removed, and only

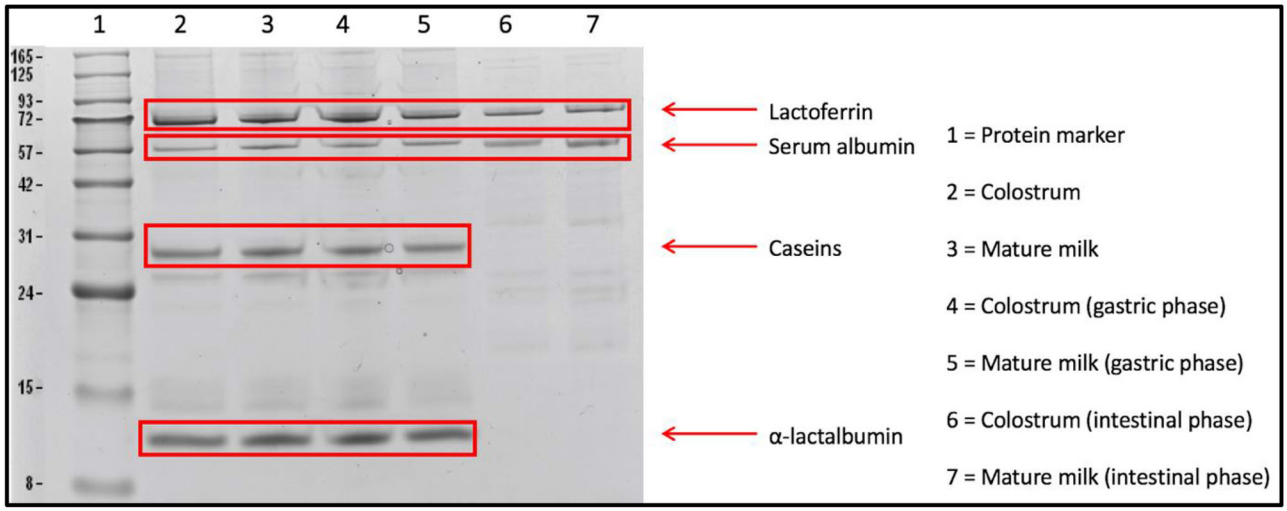

FIGURE 2 | SDS-PAGE gel showing the *in vitro* digestion of colostrum (week 1) and mature milk (week 4) of one mother, highlighting lactoferrin, serum albumin, caseins, and α-lactalbumin. The total amount of protein loaded on the gels was 20 μg per sample.

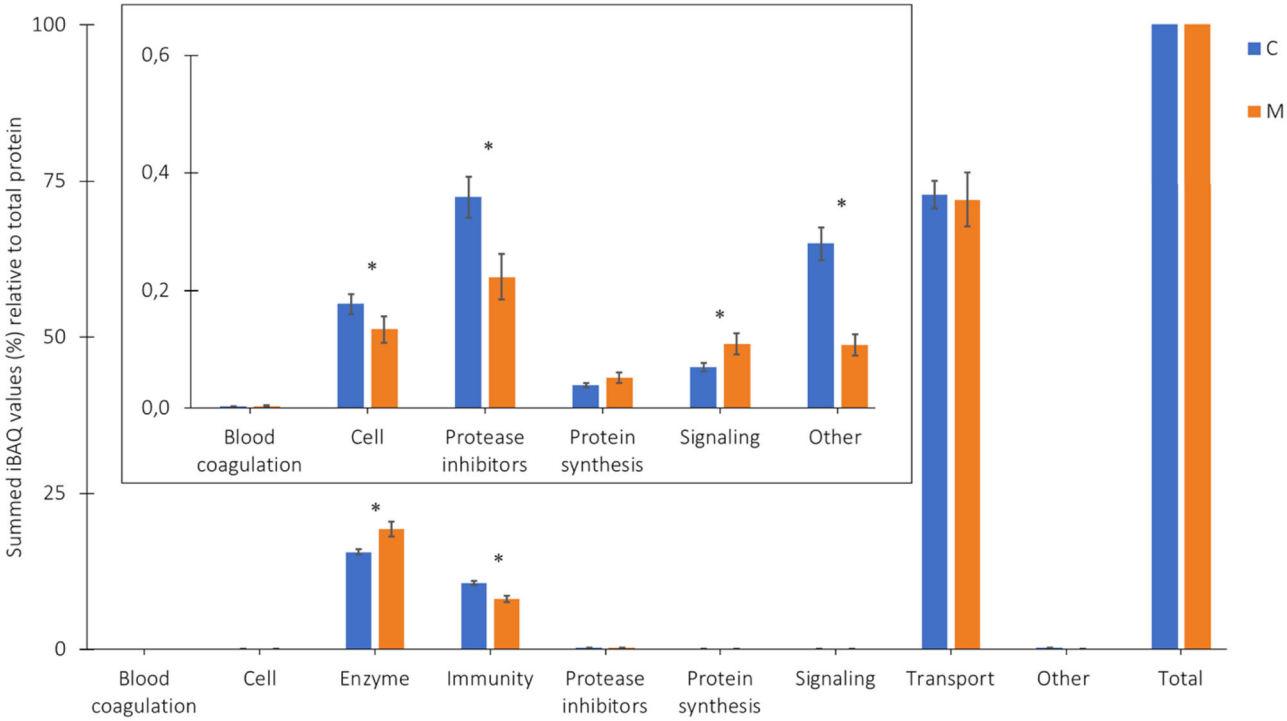

FIGURE 3 | Proportion of the protein groups (%) relative to total protein, based on the summed iBAQ values obtained by LC-MS/MS, in colostrum (C, week 1) and mature milk (M, week 4) of 7 Chinese mothers averaged. *Corresponding *p*-values (two-tailed *t*-test, $\alpha < 0.05$). Insert illustrates zooming in on the low abundant protein groups.

the free soluble caseins remained (5). The levels of the groups of enzymes and immune-active proteins in human milk showed a similar trend as described previously (5). The relative levels of enzymes were significantly higher in mature milk than in colostrum, whereas the relative levels of immune-active proteins were significantly higher in colostrum compared to mature milk (5).

It was also reported previously that large variation in protein composition existed among mothers, between populations and over time (5). In this study, it could also be observed that the protein composition differed among mothers and over lactation (**Supplementary Figure 1**). For example for Chinese mother 5, both the relative levels of immune-active proteins and enzymes increased over lactation, whereas the relative levels

TABLE 1 | Undigested human milk proteins (%) in an *in vitro* infant (0–3 months) model, based on the summed iBAQ values obtained by LC-MS/MS, categorized per biological function, for both colostrum and mature milk of 7 Chinese mothers averaged, and for gastric and intestinal digestion.

	Colostrum (week 1)		Mature milk (week 4)	
Protein function groups	**Gastric (%)**	**Intestinal (%)**	**Gastric (%)**	**Intestinal (%)**
Blood coagulation	25	0	7	0
Cell	31	0	18	0
Enzyme	22	0	20	0
Immunity	29	2	32	3
Protease inhibitors	44	1	35	3
Protein synthesis	41	1	14	1
Signaling	27	1	12	4
Transport	30	0	17	0
Other	22	0	22	0
Total	**29**	**0.3**	**18**	**0.4**

The data has been normalized to 100% per protein group, based on pre-digestion data.

of transport proteins decreased from colostrum to mature milk (**Supplementary Figure 1**). For Chinese mother 6, immune-active proteins became relatively less abundant over lactation (**Supplementary Figure 1**).

LC-MS/MS Evaluation of the Protein Composition After Gastric and Intestinal Digestion

The relative levels of undigested protein for each protein group after the gastric and intestinal digestion phase can be found in **Table 1**, for both colostrum and mature milk. Comparing colostrum and mature milk, higher percentages of undigested protein were found in the gastric phase for most protein groups in colostrum (**Table 1**). In addition, the relative levels of undigested protein for the groups protease inhibitors and protein synthesis in colostrum were the highest during gastric digestion (**Table 1**). For mature milk, protease inhibitors and immune-active proteins were digested to a lesser extent during gastric digestion (**Table 1**).

It should be noted that a significant variation exists in protein composition from the human milk among mothers after post-intestinal treatments (**Supplementary Figure 1**), however, all samples had in common that the undigested levels were the highest for the immune-active proteins after the intestinal digestion phase (**Supplementary Figure 1**). This can also be observed in **Table 1**. Transport proteins were quite stable during the gastric digestion phase, but not during the intestinal digestion phase (**Table 1**). This might be explained by the caseins, which were the main proteins within the transport protein group. Caseins were almost completely digested after intestinal digestion, as can be observed from **Table 2**. The variation in protein digestion might be affected by the initial milk composition and protein profiles of the individual mothers. The static *in vitro* digestion model itself did not contribute to the variability in milk digestion amongst all 7 mothers, as the parameters of the model were similar for both lactation time points.

TABLE 2 | The top 15 most abundant human milk proteins after *in vitro* infant (0–3 months) digestion, categorized per function ($N = 7$).

		Colostrum (week 1)		Mature milk (week 4)	
Protein function	**Name of proteins**	**Gastric (%)**	**Intestinal (%)**	**Gastric (%)**	**Intestinal (%)**
Enzymes	α-lactalbumin	22	0	20	0
	Bile salt-activated lipase	55	3	21	1
Immunity	Lactoferrin	33	1	12	3
	Ig α1-chain c-region	12	2	84	2
	Ig λ2-chain c-region	16	6	46	6
	Ig κ-chain c-region	9	8	64	12
	PIGR	39	0	61	0
	Clusterin	46	0	43	0
	Osteopontin	36	0	14	0
	β2-microglobulin	24	0	4	0
Protease inhibitor	α1-antichymotrypsin	53	1	40	1
Transport	Serum albumin	45	2	32	3
	β-casein	29	0	15	0
	α_{S1}-casein	39	0	2	0
	κ-casein	37	0	23	0
Based on top 15	**Total serum proteins**	33	2	37	2
	Total caseins	35	0	13	0

The data has been normalized to 100% per protein.

The Effect of the Higher Levels of Protease Inhibitors and Total Protein Content in Colostrum on Protein Digestion

The variation in levels of protease inhibitors and total protein were investigated in relation to the level of undigested protein, as both were hypothesized to reduce overall proteolysis. It can be observed that more intact proteins from colostrum (29%) can be found after gastric digestion compared to mature milk (18%) (**Table 1**). This effect is not expected to be related to the level of protease inhibitors, as no pepsin inhibitor has been found in human milk (5). Large fragments of proteins and undigested proteins leaving the stomach may be relevant *in vivo*, as they may still be biologically active before they are further degraded into smaller peptides, amino acids, and finally absorbed.

In addition, some undigested proteins could still be found after intestinal digestion (**Table 1**). The total protein content after intestinal digestion was similar: with 0.3 and 0.4% remaining for colostrum and mature milk, respectively (**Table 1**). Protease inhibitors were not completely digested during intestinal digestion, with 1 and 3% remaining for colostrum and mature milk, respectively (**Table 1**). In addition, it can be observed in **Figure 1**, that protein digestion varies between mothers, with no indications that higher total protein content in colostrum when compared to protein content in mature milk influenced

the level of protein digestion. The degradation of human milk proteins during the gastric and intestinal digestion phase could not be explained by the higher levels of protease inhibitors and total protein content in colostrum than mature milk, as the r-squared values were ranging between 0 and 0.3 (**Supplementary Figure 2**), and as seen above (**Figure 1** for total protein).

The Survival of Individual Human Milk Proteins in an Infant *in vitro* Gastric Digestion

It can also be observed in **Figure 2** that some human milk proteins remained partially intact after *in vitro* infant intestinal digestion. **Table 2** is based upon the 15 most abundant proteins in Chinese human milk. The other human milk proteins still present intact after intestinal digestion can be found in **Supplementary Table 1**, but not in all cases for both lactation periods.

The results showed that both milk serum proteins and caseins were not completely digested during the gastric digestion phase: with 33 and 35% remaining for colostrum and 37 and 13% remaining for mature milk, respectively (**Table 2**). Interestingly, the levels of undigested caseins after gastric digestion were higher for colostrum than for mature milk (**Table 2**). The ratio between milk serum proteins and caseins in our study ranged from 33:67 to 38:62 over lactation, respectively (data not shown). The findings in **Table 2** might be attributed to the hypothesis that lower levels of caseins in mature milk become easier to digest in the gastric phase than in colostrum.

The reason for the high level of gastric casein digestion (**Table 2**), but still partial survival of caseins, may be due to the low pepsin activity. Pepsin exerts its maximum activity at a pH of 2 (23). Another reason for this phenomenon may be the curd forming properties of casein, which happens at pH 5 (3, 17, 31). At neutral pH, caseins are negatively charged and soluble, whereas upon acidification toward their isoelectric point (4.6), as is happening under infant gastric conditions, they become less negatively charged after which caseins start aggregating into a curd. Due to the low casein level and different micelle properties in human milk, a softer curd is formed. Still, this curd is difficult to digest, which might result in a lower gastric casein digestion (11). Caseins can be degraded more easily by enzymes during intestinal digestion due to their flexible non-compact structure and lack of curd formation at pH 7 (6–8). As mentioned, many milk serum proteins survived in the gastric phase (**Table 2**). For example lactoferrin, which can appear in an iron-rich form, hololactoferrin, and an iron-free form, apolactoferrin. The iron-rich form of lactoferrin might stabilize the protein structure above pH 4, making hololactoferrin more resistant against enzymatic degradation in an infant *in vitro* gastric digestion. Previous studies have confirmed that lactoferrin is resistant against digestion in the infant's gastrointestinal tract (17, 24, 25), although using different *in vitro* digestion models. Another example, α-lactalbumin in colostrum and mature milk is not fully digested with 22 and 20% remaining, respectively (**Table 2**), which might be due to the fact that the gastric pH is too high to form the molten globule structure (3). On lowering the pH to 3 in another *in vitro* model, the acidic side chains were protonated and α-lactalbumin adopted the molten globule state, which is a less compact conformation that was easier to digest by pepsin (3). With regard to the immunoglobulins, the heavy and light chains of the different immunoglobulins are connected via disulfide bridges in human milk, making them more resistant against digestive enzymes (18), which might explain their low gastric digestion (**Table 2**). The reason for the large differences in rate of digestion between immunoglobulins from colostrum and mature milk remains unclear.

The Survival of Individual Human Milk Proteins After Infant *in vitro* Intestinal Digestion

After the gastric phase, the human milk proteins where further hydrolyzed in the intestinal phase. It can be observed in **Table 2** that some of the highly abundant milk serum proteins (lactoferrin, bile salt-activated lipase, immunoglobulins, α_1-antichymotrypsin, serum albumin) from both colostrum and mature milk were still partially present after intestinal digestion (range: 1–12%) (**Table 2**). These specific milk serum proteins were always present in all the individual intestinal digesta samples (**Supplementary Table 1**). Other abundant milk serum proteins, α-lactalbumin, PIGR, clusterin, osteopontin, β_2-microglobulin, and the 3 caseins (β-, α_{S1}–, and κ-casein) from both colostrum and mature milk were almost completely digested after *in vitro* intestinal digestion (**Table 2**). These proteins were absent in the intestinal digesta samples of most ($N = 6$) mothers (**Supplementary Table 1**). Thirty-seven other human milk proteins were found after intestinal digestion (**Supplementary Table 1**), although, the survival levels of these proteins varied between colostrum and mature milk during digestion. Among these 37 milk serum proteins, several low abundant immunoglobulins can be found after intestinal digestion (**Supplementary Table 1**), but also e.g., lysozyme, α_1-antitrypsin, and fatty acid-binding protein. Some of the milk serum proteins surviving digestion are known to be glycosylated, a feature that may have protected them against breakdown. However, as we did not analyze the level of glycosylation directly, we cannot determine whether it played a role during *in vitro* infant protein digestion.

Lactoferrin, lysozyme, immunoglobulins, antichymotrypsin, α_1-antitrypsin, and serum albumin were previously found in the infant's feces (16), even surviving fermentation. The proportion of intact human milk proteins found in the feces varied with the age of the infants, and about 10% of the total protein intake of the breastfeed infants was undigested and appeared in the feces during the early neonatal period up to 1 month of age, while only 3% was found at 4 months of age (16). The findings in **Figure 1** and **Table 1** are in the same range (16). From the current study, it became clear that overall, more than 40 milk serum proteins, including several immune-active proteins (e.g., lactoferrin, immunoglobulins) and protease inhibitors (e.g., α_1-antichymotrypsin and α_1-antitrypsin), were still partially present intact after *in vitro* intestinal digestion. These protease inhibitors

might be involved in supporting the infant's digestive tract against pathogens, as they may protect the immune-active proteins from breakdown (16, 24). Additionally, it has been reported that these protease inhibitors may affect the immune systems directly, amongst others through regulation of the complement pathway (20–22).

Undigested immunoglobulins after gastric and intestinal digestion might be important for infants in the first months of life, and might provide additional protection when the infant's immune system and digestive tract is not yet fully developed (1). This might also account for the other immune-active proteins, which survive gastric digestion, as they might still be biologically active before being further degraded during intestinal digestion. PIGR, clusterin, osteopontin, and β_2-microglobulin are highly abundant in human milk and exert important functions for the development of the infant's immune system (5). Bile salt-activated lipase and serum albumin, which are highly abundant in human milk, were also found to be resistant (relative levels ranging from 3 to 1%) against gastrointestinal enzymes from both colostrum mature milk, and able to survive intestinal digestion in an *in vitro* infant model.

CONCLUSIONS

This study provided, for the first time, detailed information on the digestion of proteins in an *in vitro* digestion model adapted for 0–3 months infants using both colostrum and mature milk from 7 individual Chinese mothers. LC-MS/MS was used to provide a more complete overview of the human milk protein composition before, during and after *in vitro* infant digestion. Protein digestion levels varied for the milk from the individual mothers. Large variation in total undigested protein was found between mothers after the gastric phase. Colostrum and mature milk were digested after the intestinal phase to a similar extent. In contrast to expectations, the extent of protein degradation was not directly influenced by protease inhibitors and the total protein content. Caseins were digested to a larger degree after intestinal digestion than most milk serum proteins. The relative levels of the immune-active milk serum proteins were overall the highest after intestinal digestion. The resulting intact immune-active milk serum proteins, like antibacterial proteins, might support the infant's intestine against pathogens.

DATA AVAILABILITY STATEMENT

The raw LC/MSMS data collected for this study are owned by the funder (Yili Industrial Group Company). Interested researchers can request a copy of this data by contacting the corresponding author (email: kasper.hettinga@wur.nl; tel.nr. +31-317-482401).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Chinese Ethics Committee of Registering Clinical Trials (ChiECRCT-20150017). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ME, HS, and KH conceived and planned the experiments. ME carried out the experiments and statistics and took the lead in writing the manuscript. SB contributed to sample preparation and analysis. All authors contributed to the interpretation of the results and provided critical feedback by reviewing the manuscript.

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SUPPLEMENTARY MATERIAL

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The Role of Milk Components, Pro-, Pre-, and Synbiotic Foods in Calcium Absorption and Bone Health Maintenance

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Increasing peak bone mass during adolescence and reducing bone loss in later life are two approaches to reduce the risk of osteoporosis with aging. Osteoporosis affects a large proportion of the elderly population worldwide and the incidence is increasing. Milk consumption is an accepted strategy in building peak bone mass and therefore may reduce the risk of osteoporosis. In childhood calcium, phosphorous, and growth factors are the important components to support bone growth but in adults the positive influence on bone density/maintenance may also be due to other bioactive proteins/peptides or lipids in milk acting directly in the gastrointestinal tract (GIT). Lactose has been known to increase calcium absorption; galactooligosaccharides (GOS) are derived from lactose and are non-digestible oligosaccharides. They have been shown to improve mineral balance and bone properties as well as causing increases in bifidobacteria in the gut, therefore a prebiotic effect. Supplementation with fortified milk and dairy products with added prebiotics, increased both calcium and magnesium absorption and caused some modulation of gut microbiota in animals and humans. Fermented milk is now also recognized to contain highly active components such as vitamins, peptides, oligosaccharides, and organic acids. In this review, the role of milk and milk components in improving calcium absorption and thereby supporting bone health is discussed. In addition, some reference is made to the significance of combining the inherent beneficial components from milk with fortificants/nutrients that will support bone health through adulthood. Novel data suggesting differences in diversity of the microbiota between healthy and osteoporotic women are provided.

Keywords: milk, probiotics, prebiotics, calcium absorption, bone health, gut microbiome

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INTRODUCTION

Osteoporosis is a condition of skeletal fragility characterized by decreased bone mass and microarchitectural deterioration of the bone structure (1), with the consequence of an increased risk of fracture. Several factors can influence the development of osteoporosis with aging. Genetic background and ethnicity are non-modifiable determinants of bone health while nutrition and lifestyle are modifiable factors that can contribute to osteoporosis. Peak bone mass (PBM) is the greatest amount of bone an individual can attain and is reached in the late teens and early 20's.

At the age of 18 usually 90% of PBM is reached and while nutrition and exercise have significant effects on the attainment of PBM, genetics has the strongest influence (2). While calcium is the most important nutrient for bone growth and reduction of bone resorption, the use of dairy products or milk may have further benefits due to bioactive components of milk and milk or dairy foods are also excellent vehicles for fortification. In this review the role of milk in increasing bone growth and reducing bone resorption is discussed followed by a review of further bioactive components of milk which may enhance the bone health benefits of milk/dairy.

MILK AND BONE HEALTH IN CHILDREN AND OLDER ADULTS

Several randomized controlled trials in children and adults have used milk and milk products as the principal source of calcium, all showing positive effects (1). Early studies in Caucasian and Gambian children indicated that increasing milk intake may increase bone formation, and increase the levels of insulin growth factor—one (IGF-I), while supplementing with fortified milk with additional vitamin D resulted in significant increases in lumbar spine bone density compared to the controls (3). In contrast a meta-analysis as well as a review indicated that increased milk/ dairy intake may only be effective in children with a low habitual intake (2). A prospective study in children aged 5–10 years over 1 year however, reported a marginal significant effect on whole body (WB) bone mineral content and improvement in the WB *z*-score (2).

Iuliano and Hill (3) provided a critical appraisal of the evidence for a beneficial effect of dairy foods on bone density/ bone biomarkers throughout the lifespan. Several studies have shown significant effects of regular or fortified milk on bone biomarkers reducing bone resorption, but evidence of significant effects on bone density and reduction in fracture risk are less available. As discussed by Iuliano and Hill (3), to date no randomized controlled prospective studies have been executed on milk/ dairy foods investigating the anti-fracture effects. In general studies using either calcium supplementation or dairy foods had several flaws such as poor compliance, insufficient numbers of participants, shorter duration of the studies, and a significant drop-out rate which then reduced the opportunity to observe an antifracture effect. Observing a change in bone density or reduction in fracture risk may require a specific population to be studied and a longer trial period which may not be feasible or affordable.

COMPONENTS OF MILK AND DAIRY FOODS

The main components of dairy that have been shown to affect the human health are protein (from whey, casein, fragments), fat [saturated/unsaturated, short chain fatty acids (SCFA)], minerals (calcium, magnesium, phosphate), sodium, and sugars (lactose, galactose, glucose) as well as the components of the milk fat globule membrane (MFGM) (i.e., the biological membrane surrounding the lipid droplets in milk) (4). The final dairy product nutritional value tends to be determined by the milk-based source (age, mammalian-type, feed and lactation stage), processing/preparation type (storage condition, temperature and heat duration) as well as the fermentation and cultures used and any additives (5). **Table 1** shows a list of controlled trials using dairy products to mitigate bone loss and support bone health in adults.

The health-promoting benefit and effects of probiotic, prebiotic and synbiotic food products have been researched mainly in animal models however, not much work has been done with humans. Research shows that probiotics, prebiotics and synbiotics can be beneficial for mineral absorption and bioavailability in bone health, modulation of growth factors, reduction in inflammatory cytokines and an antiarthritic effect, blood lipids and a protective effect on chronic inflammatory conditions such as gastritis, Crohn's disease, inflammatory bowel disease (IBD) and type II diabetes (15).

THE IMPACT OF MILK COMPONENTS (MILK PROTEIN, MILK FAT AND MILK SUGAR) ON BONE HEALTH

Milk Sugars

Milk is known as an important source of energy (from the milk sugars), high-quality protein and essential vitamins as well as minerals. Lactose malabsorption and/or intolerance have been linked to bone loss and fractures (16). D-galactose has been linked with inducing aging, oxidative stress and inflammation; however galactooligosaccharides and transgalactooligosaccharides are known to stimulate calcium absorption in humans (17) which is important for bone health. Lactose is a disaccharide carbohydrate which is a well-known nutrient that promotes intestinal calcium absorption in mammals. Hydrolyzed lactose forms simple sugars, glucose and galactose with organic acids which reduce the pH thereby enhancing the transport and absorption of calcium ions as calcium gluconate in the intestine (18). Meanwhile, if unhydrolyzed, lactose moves into the large intestine acting as a prebiotic where it stimulates the growth of health-promoting microbes such as *Bifidobacteria* (19). Lactose has also been reported to enhance the formation of non-digestible oligosaccharides (NDOs) such as transgalactooligosaccarides due to lactic acid bacteria growth.

Milk Fats

Research into the effects of fatty acids on human health has identified that diets high in palmitic acids (C16:0), myristic (C14:0) and lauric (C12:0) are associated with increased risk of atherosclerosis (20), coronary heart diseases and obesity (21). However, the fat globules of milk contain short-chain and polyunsaturated fatty acids. Unsaturated fatty acids are regarded as "healthy fats" due to their impact on cholesterol levels in the blood (22). Polyunsaturated fatty acids (PUFAs) are known to have a more beneficial effect on cholesterol levels compared to MUFAs (23).

TABLE 1 | Controlled trials on the effect of dairy products on bone turnover markers and bone mass[a].

Type of dairy	Number of subjects	Age[b]/Sex	Duration (months)	Outcome	References
Fortified milk and yogurt, 3 servings per day	101	60.5 ± 0.7/F	12	Reduced PTH and CTX, increase in BMD	(6)
Milk	30	59.3 ± 0.3/F	1.5	Reduced PTH, CTX, PINP and osteocalcin	(7)
High-protein dairy	130	45.6 ± 8.9/F/M	12	Attenuated bone loss	(8)
Fortified fermented milk (175mL)	85	58.7 ± 0.3/F	0.5	Reduced excretion of nocturnal deoxypyridinoline	(9)
Skimmed soft cheese, 2 servings/d	37	84.8 ± 8.1/F	1	Reduced PTH, CTX and TRAP 5b, and increased 25(OH)D and IGF-1	(7)
Fortified milk	120	>55/F	4	Reduced PTH, CTX, PINP and osteocalcin	(10)
Milk (2 × 500 mL/d)	20	22.4 ± 2.4/F	3	Reduced PTH and CTX	(11)
Fortified milk	63	>55/F	3	Reduced CTX	(12)
Skimmed soft cheese, 2 servings (100 g/d)	71	56.6 ± 3.0/F	1.5	Reduced PTH, CTX and TRAP 5b, and increased IGF-1	(13)
Vitamin D and Calcium-fortified yogurt	89	85.5 ± 6.6/F	2	Reduced PTH, CTX and TRAP 5b	(14)

[a]*BMD, bone mineral density; PTH, parathyroid hormone; CTX, cross-linked telopeptide of type 1 collagen; PINP, procollagen type I N-propeptide; TRAP 5b, tartrate-resistant acid phosphatase; 25(OH)D, 25-hydroxyvitamin D.*
[b]*Mean ± SD.*

Reports has suggested that n−3 PUFA prevents cardiovascular disease and improve the immune response. Likewise, the anticancer and anti-atherogenic properties of oleic acid (C18:1) and linolenic acid (C18:3) have been reported (20). Research into the beneficial effects of n-6 PUFA has shown it ability to improve sensitivity to insulin thereby reducing the incidence of type 2 diabetes (24). Furthermore, the health-positive properties of conjugated linoleic acid cis9trans11 C18:2 (CLA) have been reported which include anticarcinogenic (25), anti-atherosclerotic (26), antioxidative (27) and anti-inflammatory effects (28). In addition, Lim et al., elucidated the anticancer and anti-atherosclerotic effects of trans-vaccenic acid, the main trans-C18:1 isomer in milk fat (29). CLA has been shown to be positively associated with bone mass in mice (30) and postmenopausal women (31).

Milk Proteins

It is well-known that milk has more beneficial effects on bone health when compared to other food products. Milk is made up phosphoproteins such as growth factors, immunoglobulins, bovine serum albumin (BSA), alpha-lactalbumin and beta-lactoglobulin and a large quantity of caseins (32). Milk proteins are made up of bioactive peptides which are inactive or latent until released and activated by the process of enzymatic proteolysis during food processing, gastrointestinal digestion, fermentation of milk with proteolytic starter cultures or hydrolysis by proteolytic enzymes (33). Enzymatic proteolysis, for example, the actions of proteinases and peptidases from lactic acid bacteria are important for the release and activation of the milk-protein derived bioactive peptides known to be inactive within the sequence of the parent protein (34).

Casein is the major source of amino acids in the milk-peptide sequence. The active sequence size which may vary from 2 to 20 amino acid residues and many peptides are also known for its multi-functional properties (33). Digestive enzymes in the human gastrointestinal tract (GIT) contribute to the further breakdown of long casein-derived oligopeptides which may lead to the release of bioactive peptides. Casein phosphopeptides (CPP) prepared from cow's milk beta-casein is known to enhance the absorption of calcium by increasing the soluble calcium concentration in the small intestinal lumen (35). On liberation, these bioactive peptides may produce hormone-like activity acting as regulatory compounds (34). Milk whey protein, in particular the milk basic protein (MBP) fractions have also been shown to promote bone formation and increase bone mineral density in both *in vivo* and *in vitro* studies (36, 37). Caseinoglycomacropeptide (CGMP) is an acidic protein from milk released from kappa-casein during coagulation in the making of cheese. The milk acidic protein has also been reported to reduce bone loss in ovariectomised rats (38, 39). Lactic acid bacteria produce lactic acid whey by the process of fermentation in the making of cheese. However, the lactic acid whey and mineral acid whey do not contain CGMP (32).

Lactoferrin is known as an iron-binding glycoprotein found in milk which is capable of stimulating bone formation cells, osteoblasts while inhibiting the osteoclasts, bone resorption cells (40). Studies conducted *in vitro* (41) and *in vivo* (42) have shown a positive association and effects on bone mass in ovariectomized rats and mice. Osteopontin is another highly glycosylated and phosphorylated protein typically found in milk. It is a non-collagenous protein in bone which protects the exposed bone surface. Osteopontin is known to be important for bone cells and cell-matrix interactions (32).

PRO-, PRE-, AND SYNBIOTICS

Milk products such as yogurt and kefir contain probiotics, however milk and dairy products may also be considered a good carrier of probiotics and prebiotics which are important for calcium absorption. Calcium bioavailability depends on various factors such as the source of calcium, age, transit time, the amount of calcium ingested, intestinal content, and type of diet. All of these factors play a role in the rate of calcium absorption. Calcium is a divalent cation which occurs as salt in food. Calcium gets absorbed in soluble form and must be in an ionized form (43). Prebiotics for example, improve mineral balance and bone properties by lowering cecal pH which can increase calcium solubility and absorption, increase in cecal wall thickness and content weight as well as increases in bifidobacterial (43).

Benefit of Probiotics

Probiotics are known to contain living microorganism that tends to exert beneficial effects onto the GIT microbial population. The most commonly defined probiotic microbes are member of the genera *Lactobacillus*, *Bifidobacterium* as well as lactic acid and non-lactic acid bacteria (44). Probiotics has numerous advantages and functions in the human organism with the main advantage being the ability to maintain appropriate balance between pathogens and bacteria necessary for normal function. This positive effect is also used to restore the natural microbiota in the gut after antibiotic therapy (45).

Another role of the probiotics is in the counteraction of the activities of pathogenic intestinal bacteria which were caused by contaminated food and environment. Probiotic microbes have been reported to increase the immune system efficiency, enhance vitamin, and mineral absorption as well as generate organic acids and amino acids (46). Some probiotics may produce enzymes such as lipase and esterase and co-enzymes A, Q, NAD and NADP. All these have been shown to exhibit antibiotic (acidophiline, bacitracin and lactacin, a bacteriocin) (47), anti-cancerogenic (48), and immunosuppressive properties (49).

Bone health depends on the bone remodeling cycle and the balance of the bone formation by osteoblasts and bone resorption process by osteoclasts. These processes is regulated by hormones, immune cells, and the gastrointestinal system while the healthy intestine is known for mineral (calcium, phosphorus) absorption and subsequent bone mineralization (50). In addition, the intestine also produces endocrine factors such as incretins and serotonins that signal (crosstalk) to bone cells.

A randomized control trial have shown that *Lactobacillus reuteri* reduced bone loss in elderly women aged 75–80 years with low bone mineral density in Sweden (51). Similarly, a multispecies probiotic supplement (Gerilact capsule) slowed down the rate of bone turnover in postmenopausal women aged 50–72 years in Iran (52). Furthermore, when the probiotic is combined with bioavailable isoflavones, it improved estrogen metabolism and bone health status in postmenopausal women (53).

Benefit of Prebiotics

Prebiotics are also known as functional food components that have a great potential of modulating the gut microbiota as well as the gut environment and pH. These non-digestible oligosaccharides containing prebiotic activities occur naturally in human milk and plants. Prebiotics either occur naturally in plant-based foods or by enzymatic conversion of sugars from synthetic production of carbohydrate structures or soluble dietary fibers (54).

Some prebiotics are artificially produced, they include lactulose, galactooligosaccharides (GOS), fructooligosaccharides (FOS), maltooligosaccharides (MOS), cyclodextrins, and lactosaccharose. Lactulose contains a significant part of oligosaccharides produced and can be as much as 40% content (55). Fructans, such as inulin and oligofructose, are believed to be the most used and effective in relation to many species of probiotics (46). Prebiotics in the colon are fermented by beneficial bacteria. This fermentation which may then lead to an increased production and/or a change in the relative abundance of SCFAs (acetate, butyrate and propionate) and lactate, moderate reduction in the colonic pH, increase in stool mass, reduction in nitrous end products and fecal enzymes, and a resulting improvement in the immune system (56). There are two mechanisms by which dietary fiber increases the fecal bulk: incompletely fermented types of fiber binding to water throughout the GIT, whereas the readily fermented types of fiber tend to increase the microbial mass (57).

Human studies have shown FOS (58), GOS (59) and xylooligosaccharide (XOS) (60) are compounds that are able to increase the proportion of *Bifidobacterium* in the GIT whereas the number of *Bacteroidaceae* and *Clostridium perfringens* are decreased. Another study with the intake of lactulose ($2 \times 10\,g\ d^{-1}$) in a rat model showed increased probiotic bacteria and decreased putrefactive and potential pathogenic bacteria (61).

Benefit of Synbiotics

Bacteria are known to produce genes in response to changes in their environment. These genes are responsible for encoding enzymes that produce metabolites such as short chain fatty acids (i.e., butyrate, lactate, propionate and acetate), branch-chained fatty acids, bile acid derivates and vitamins. These metabolic products are however dependent on the availability of substrate produced in part by prebiotics and the intestinal microbiota (62). Furthermore, prebiotics causes the lowering of intestinal pH and is also important for maintaining the osmotic retention of water in the bowel (46).

These synbiotics are also known to stimulate and result in enlarged absorption surface area by the proliferation enterocytes mediated by bacterial fermentation (63). Part of the activities of these microbes includes absorption and release of essential nutrients from the diet (mainly calcium and phosphate), regulation of the immune system, support of intestinal integrity and function as well as the ability to repress pathogenic invasion of harmful bacteria or colony (62). It also responsible for promoting the expression of calcium-binding proteins and degradation of mineral complexing molecules such as oxalates and phytic acid. The commensal bacteria have recently been

also thought to affect the bone physiology through production of small molecules such as serotonin or estrogen-like molecules beneficial for osteogenesis.

A study conducted in healthy postmenopausal women showed that synbiotic fermented milk was capable of enhancing the oral bioavailability and plasma concentrations of isoflavones (64). Isoflavones are known to be able to alleviate the risk of osteoporosis.

DISCUSSION

In this review of the literature, we have evaluated the benefits of milk components, probiotics, prebiotics and synbiotics in calcium absorption and bone health. Dairy products especially milk is known to provide phosphorus intake simultaneously with calcium intake unlike supplementation. Likewise, milk/dairy do not contain calcium absorption inhibiting substances such as oxalates, phytates or polyphenols (65). Milk contains substantially high levels of vitamin D, riboflavin, vitamin C and vitamin B12 necessary for bone health maintenance. The consumption of milk is also known to influence hormones such as IGF-1 and the bone cytokines.

According to Goulding (66), CPPs increase calcium absorption by reducing the production of insoluble calcium phosphate complexes through signaling and biomodulatory effects in the GIT. In a systematic review of literature by Kouvelioti et al. (67), they concluded that the systematic consumption of dairy products may be beneficial for bone structure and development. Furthermore, a review by Heuvel and Steijns (68) revealed a strong evidence for the positive impact of calcium from dairy products with or without vitamin D on bone mineralization. Studies have also shown that calcium absorption may be mediated by the gut microbiota specifically by *Bifidobacteria* and *Lactobacillus*, and probably fermented milk products (69).

Microbial Differences Between Healthy and Osteopenic/Osteoporotic Women

To our best knowledge, two human studies have investigated the role of gut microbiota in bone health. The two studies used 16S ribosomal RNA (rRNA) gene sequencing to characterize the microbiota composition profile. Wang et al. (70) found the relative abundance of *Blautia*, *Parabacteroides,* and *Ruminococcaceae* to be higher among the osteoporotic patients compared to their normal controls. This study was conducted with a small sample size of mainly female patients recruited from a Chinese hospital with mean age between 64 and 70 years. Das et al. (71) found that *Lactobacillus* and *Bacillus* was proportionally more abundant in participants with osteoporosis compared to those with normal BMD. This study involved 181 female and males aged between 55 and 75 years care referrals.

Our recent novel findings using shotgun metagenomics have shown alpha diversity differences between healthy (H) and osteopenic/osteoporotic (OP) postmenopausal women in New Zealand. The women were classified according to the World Health Organization (WHO) *T*-score cut-offs for diagnosing osteoporosis. The findings also showed a higher proportion of *Bacteroidetes Bacteroides* among the OP group compared to the H group. The result of the finding also indicated higher microbial diversity among the H group than the OP group. Lower microbial composition diversity in the women with bone disorders is in agreement with other studies where lower diversity has been observed in "disease" states vs. healthy controls. Limited published epidemiological evidence supports the relationship between the gut microbiota and bone mineral density and robust human studies are required to evaluate this relationship. Our results provide a basis for a longitudinal intervention assessment of the role of intestinal microbiota composition and function in osteoporosis to yield preventive and therapeutic interventions. In addition, further investigations using probiotic, prebiotic, or synbiotic foods in intervention studies may be able to provide more insight into the role of these foods in bone health maintenance.

CONCLUSION

Milk and milk components have been shown to significantly affect growth and bone mineralization in children. Studies in older women using regular or fortified milk have reported significant changes in bone biomarkers and some changes in bone mineral density, though not reduction in fracture risk. Milk and dairy products also contain several other bioactives that can affect bone formation or resorption. Combining the inherent bioactives in milk with additional pre- or probiotics can enhance the beneficial effects of milk on bone health via improvement of the intestinal absorption of minerals especially calcium and magnesium. The observed differences in diversity of microbiota between healthy and osteoporotic women open new avenues for modulating the gut profile and thereby preventing bone loss or maintaining healthy bone density.

AUTHOR CONTRIBUTIONS

MK was responsible for the conceptualization, writing the abstract, introduction and conclusion while BI-O wrote the body of the article. Both authors read, reviewed, and approved the final draft.

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REVIEW
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Composition, Structure, and Digestive Dynamics of Milk From Different Species—A Review

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Background: The traditional dairy-cattle-based industry is becoming increasingly diversified with milk and milk products from non-cattle dairy species. The interest in non-cattle milks has increased because there have been several anecdotal reports about the nutritional benefits of these milks and reports both of individuals tolerating and digesting some non-cattle milks better than cattle milk and of certain characteristics that non-cattle milks are thought to share in common with human milk. Thus, non-cattle milks are considered to have potential applications in infant, children, and elderly nutrition for the development of specialized products with better nutritional profiles. However, there is very little scientific information and understanding about the digestion behavior of non-cattle milks.

Scope and Approach: The general properties of some non-cattle milks, in comparison with human and cattle milks, particularly focusing on their protein profile, fat composition, hypoallergenic potential, and digestibility, are reviewed. The coagulation behaviors of different milks in the stomach and their impact on the rates of protein and fat digestion are reviewed in detail.

Key findings and Conclusions: Milk from different species vary in composition, structure, and physicochemical properties. This may be a key factor in their different digestion behaviors. The curds formed in the stomach during the gastric digestion of some non-cattle milks are considered to be relatively softer than those formed from cattle milk, which is thought to contribute to the degree to which non-cattle milks can be easily digested or tolerated. The rates of protein and fat delivery to the small intestine are likely to be a function of the macro- and micro-structure of the curd formed in the stomach, which in turn is affected by factors such as casein composition, fat globule and casein micelle size distribution, and protein-to-fat ratio. However, as no information on the coagulation behavior of non-cattle milks in the human stomach is available, in-depth scientific studies are needed in order to understand the impact of compositional and structural differences on the digestive dynamics of milk from different species.

Keywords: milk, composition, digestion, curd, protein, fat, structure, stomach

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INTRODUCTION

Milk has evolved to meet the nutritional and physiological requirements of the neonate. Milk is thus regarded as a high-quality food, nutritionally. Humans are known to have consumed cattle (*Bos taurus*, cow) and non-cattle (such as goat and sheep) milks as part of their diet since prehistoric times (1, 2). As a convenient source of nutrition, cattle milk is the most-consumed milk worldwide because of its widespread availability and large production volumes. Non-cattle milks are of nutritional importance to people in developing countries as well as in geographical areas in which the natural climate is unsuitable for the survival of dairy cattle (3, 4). For example, buffalo milk in Asia, sheep milk in Europe and the Mediterranean basin (including the Middle East), camel milk ("the white gold of the desert") in Africa, goat milk ("the cattle of the poor") in Africa and southern Asia, horse milk in the steppe areas of central Asia, yak milk on the Tibetan plateau, reindeer milk in northern Scandinavia, musk ox milk in the Arctic, and mithun milk in the hilly regions of the Indian subcontinent (3, 5).

Of the total world milk production, the proportion of total non-cattle milk production has increased from ∼9% in 1961 to 19% in 2018 (**Figure 1**). Of the total global non-cattle milk production, buffalo milk has nearly tripled, camel milk has nearly doubled, and goat milk has slightly increased during this period. No world statistics on the amounts of milk produced from other dairy species, such as yak, horse, donkey, deer, musk ox, and llama, are available. Much of the non-cattle milk production remains officially unreported because of the unknown amounts that are consumed locally at a farmer's home or are sold directly by farmers to local people, especially in developing countries (6, 7).

The addition of milk as a product to non-cattle farm systems adds value and helps farmers in dealing with the fluctuating prices of meat, hair, and wool. The buffalo, goat, sheep, and camel milking industry is well set-up in many parts of the world, is gaining popularity, and is proving to be a profitable business for those who have already implemented it. Recently, New Zealand has introduced the development of a red deer dairy farming system. Large dairy companies as well as specialized small and medium enterprises (SMEs) are also interested in using non-cattle milks as a diversification strategy for their product portfolios. The regulatory requirements to ensure the safe production of cattle milk (and milk products) are well-defined in most of the world. However, the same regulatory limits may not be true to non-cattle milk and milk products. Thus, the emphasis on species-specific regulatory standards to guarantee the safety and quality of different milk for human consumption is needed (8–11). Also, understanding the significance of compliance to religious dietary laws (such as Kosher or Halal) will be of importance to the non-cattle milk-based dairy companies for gaining acceptance of their products from the various consumer groups (12).

In recent years, the opportunities for non-cattle milk production and the manufacture of products have expanded because the numbers of dairy cattle are perceived to be reaching their limit from environmental perspectives. Non-cattle milks are also believed to have certain nutritional benefits compared with cattle milks. For example, goat, sheep, camel, horse, and donkey milk are considered to be relatively more easily digestible, less allergenic, and more similar to human milk than cattle milk (4, 13, 14). In addition, non-cattle milks can be utilized for developing high value specialized dairy products of international as well as regional (local cultural) importance, such as cheese, yogurt, butter, ghee, ice-cream, fermented milk, probiotic dairy drinks, milk tablets, infant formulas (3, 15, 16). However, relatively little scientific information on the nutritional benefits of non-cattle milks is available. In addition, there is a significant gap in scientific knowledge on the detailed compositions, especially the minor components, and the protein and lipid structures in these milks.

COMPARATIVE COMPOSITIONS OF CATTLE AND NON-CATTLE MILKS

The comparative compositions of milk from different species have been extensively reviewed in previous studies (5, 17–19). The milk from different species vary in composition (**Table 1**). Protein, fat, lactose, and minerals are the four major components in all milks, irrespective of the species (18); the composition of milk within the same species varies considerably because of various factors, such as breed, stage of lactation, milking interval, type of feed, and climate (7, 19). For example, Li et al. (26) reported recently that the stage of lactation is a key factor responsible for differences in the compositional and physicochemical properties of dairy cattle milk in a seasonal calving system in New Zealand.

Non-ruminant milks (such as those from horse and donkey) are somewhat similar to human milk (in terms of protein, lactose, and ash contents), compared with dairy cattle milk and other ruminant milks (**Table 1**). Ruminant milks have higher protein and fat contents, compared with human milk and other non-ruminant milks. Human milk contains much higher amounts of total lactose-derived oligosaccharides than milk from other species (**Table 1**). Goat milk is also known to have a relatively higher oligosaccharide content, the composition of which is considered to be similar to that of human milk (27, 28).

Proportions of Major Proteins

Compared with cattle milk and other ruminant milks, horse and donkey milk have a low casein-to-whey-protein ratio, similar to that in human milk. Among the ruminant milks, goat, sheep, and camel milk have a lower casein-to-whey-protein ratio as well as a relatively higher β-casein-to-α_s-casein ratio compared with cattle milk (**Table 2**). Thus, these non-cattle milks are an attractive alternative as a potential natural ingredient for infant formula (13); a lower casein-to-whey-protein ratio (i.e., a higher proportion of whey proteins) has been shown to be more desirable for faster digestion of the milk proteins in infant formula than a casein-dominant protein composition (31, 32). As human milk has the lowest casein-to-whey-protein ratio, has a high β-casein-to-α_s-casein ratio, and contains no β-lactoglobulin (**Table 2**), milk from other species with similar properties are of

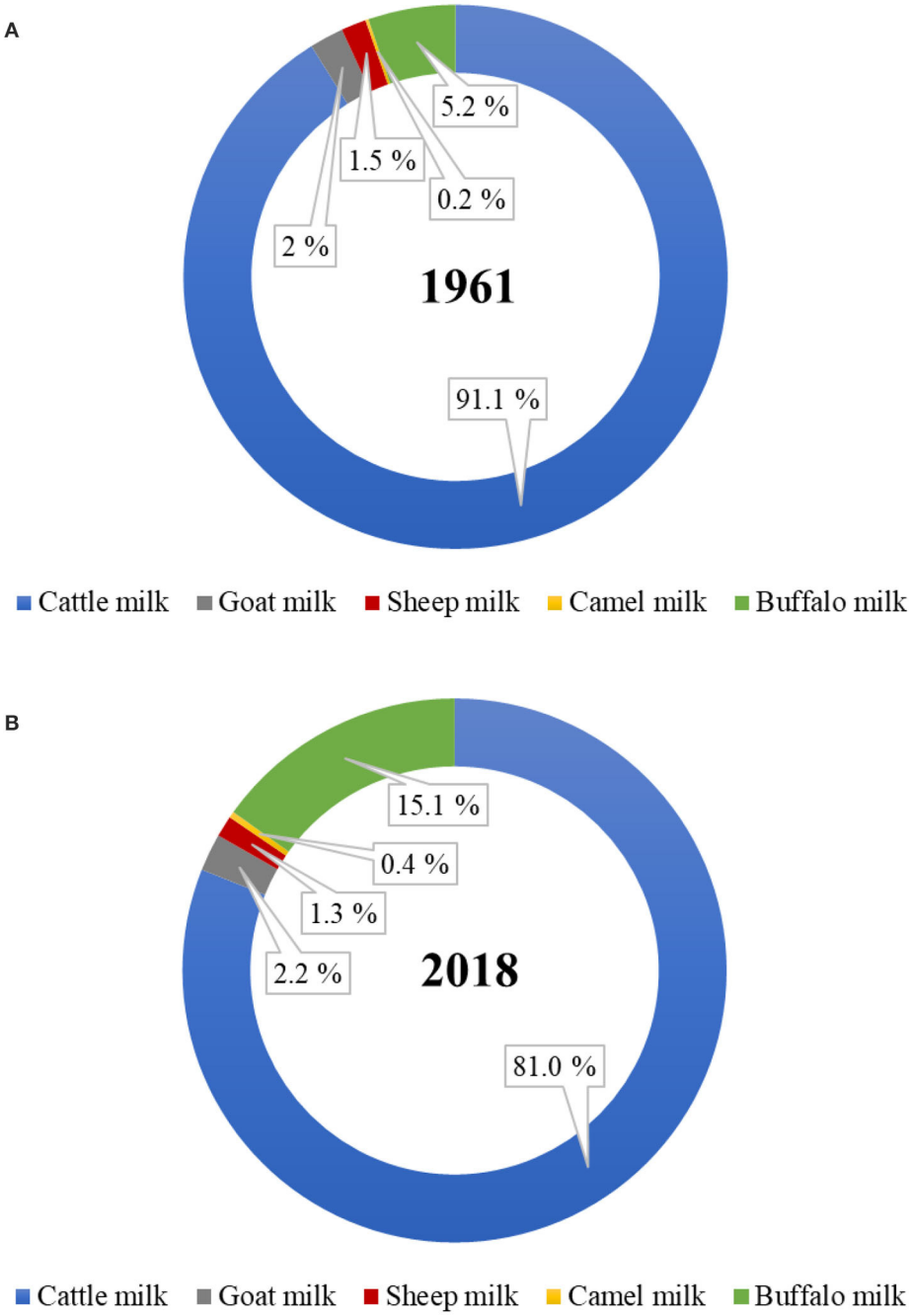

FIGURE 1 | Proportion of dairy cattle and non-cattle milks produced globally in the year **(A)** 1961 and **(B)** 2018. (*Source:* FAOstat, March 2020).

great interest to the consumer as well as to the dairy industry for the development of specialized dairy products, not only for infants but also for people in other age groups.

β-Lactoglobulin is considered to be one of the major allergens that is responsible for cattle milk allergy in children (33). Thus, milk from species that lack β-lactoglobulin or have lower β-lactoglobulin-to-α-lactalbumin ratios are of interest for human consumption. Camel milk, like human milk, does not contain β-lactoglobulin (34, 35) or it may be present in trace amounts in different forms (36–38). Llama milk is also known to contain no β-lactoglobulin (5, 39), but little detailed information on its protein composition is available.

Casein Micelle Characteristics

Individual caseins (α_{s1}-, α_{s2}-, β-, and κ-casein) are present in all milks as self-assembled particles known as "casein micelles"

TABLE 1 | General composition (g 100 mL^{-1}) of milk from different mammalian species[#].

Properties	Ruminants						Non-ruminants		Human
	Cattle	Buffalo	Goat	Sheep	Red deer*	Camel	Horse	Donkey	
Total solids	11.8–13.0	15.7–17.2	11.9–16.3	18.1–20.0	20.0–30.5	11.9–15.0	9.3–11.6	8.8–11.7	10.7–12.9
Protein	3.0–3.9	2.7–4.7	3.0–5.2	4.5–7.0	5.9–10.6	2.4–4.2	1.4–3.2	1.4–2.0	0.9–1.9
Fat	3.3–5.4	5.3–9.0	3.0–7.2	5.0–9.0	6.6-19.7	2.0–6.0	0.3–4.2	0.3–1.8	2.1–4.0
Lactose	4.4–5.6	3.2–4.9	3.2–5.0	4.1–5.9	2.6-6.2	3.5–5.1	5.6–7.2	5.8–7.4	6.3–7.0
Ash	0.7–0.8	0.8–0.9	0.7–0.9	0.8–1.0	1.04–1.18	0.69–0.9	0.3–0.5	0.3–0.5	0.2–0.3
Oligosaccharides[Ω]	0.003–0.006	No data	0.025–0.030	0.002–0.004	No data	No data	No data	No data	0.500–0.800

[#]*Source: Adapted and modified from Claeys et al. (19) and Crowley et al. (13).*

**Values based on minimum and maximum values found in the literature for different species of red deer; may include values from different stages of lactation (20–24).*

[Ω]*Values derived from Martinez-Ferez et al. (25).*

TABLE 2 | Protein profile (g L^{-1}) of milk from different mammalian species[#].

Protein fractions	Ruminants						Non-ruminants		Human
	Cattle	Buffalo	Goat	Sheep	Red deer*	Camel	Horse	Donkey	
Total casein	24.6–28	32–40	23.3–46.3	41.8–52.6[∞]	∼57–84	22.1–26.0	9.4–13.6	6.4–10.3	2.4–4.2
Total whey proteins	5.5–7.0	6	3.7–7.0	10.2–16.1[∞]	∼11–15	5.9–8.1	7.4–9.1	4.9–8.0	6.2–8.3
Casein-to-whey protein ratio	82:18	82:18	78:22	76:24	∼80:20–85:15	73:27–76:24	52:48	56:44	29:71–33:67
Major caseins									
α_{s1}-Casein	8–10.7	8.9	0–13.0	2.4[∞]-22.1	–	4.9–5.7[Ω]	2.4	Present	0.77
α_{s2}-Casein	2.8–3.4	5.1	2.3–11.6	6.0[∞]	–	2.1–2.5[Ω]	0.2	Present	Absent
β-Casein	8.6–9.3	12.6–20.9	0–29.6	15.6–39.6[∞]	–	14.4–16.9[Ω]	10.66	Present	3.87
κ-Casein	2.3–3.3	4.1–5.4	2.8–13.4	3.2–12.23[∞]	–	0.8–0.9[Ω]	0.24	Present	0.14
Major whey proteins									
β-Lactoglobulin	3.2–3.3	3.9	1.5–5.0	6.5–13.5[∞]	–	Absent	2.55	3.3	Absent
α-Lactalbumin	1.2–1.3	1.4	0.7–2.3	1–1.9	–	0.8–3.5	2.37	1.9	1.9–3.4

[#]*Source: Adapted and modified from Claeys et al. (19) and Crowley et al. (13).*

**Values (g kg^{-1}) derived from Arman et al. (20) and SDS-PAGE image analysis of our previous work (29). There are insufficient data for red deer milk in the literature to derive approximate values.*

[Ω]*Values derived from Kappeler (30).*

[∞]*Values (g kg^{-1}) derived from SDS-PAGE image analysis of our previous work (29).*

(40). The fundamental structure of the casein micelles in the milk from many species has not been studied in great detail, except in dairy cattle milk. Recently, Ingham et al. (41) used small-angle X-ray scattering and reported that the internal structures of the casein micelles of cattle, goat, and sheep milk had strong similarities with only slight differences, which may be due to the differences in casein composition, hydration, and physicochemical properties.

Apart from the differences in the proportions of different caseins (**Table 2**), the casein micelles in the milk from different species differ in size, hydration, and mineralization (**Table 3**). Among all mammalian milks, the casein micelles in human milk have the smallest diameter. The casein micelle sizes of goat, sheep, deer, camel, and horse milk are larger than that of human milk as well as cattle milk (**Table 3**). Sood et al. (53) reported that the loss of micellar calcium from the skim milk casein micelles (when dialyzed against same skim milk sample containing ethylenediaminetetraacetic acid, EDTA) resulted in increased hydration (or swelling) of casein micelles. Based on this, it was considered that the hydration level of the casein micelles was negatively correlated with mineralization of micelles (54) i.e., when the mineralization of the casein micelle increases, the degree of hydration of casein micelle decreases. Thus, the lower hydration of goat and sheep milk casein micelles had been related to its higher mineralization than those of cattle milk casein micelles (55, 56). Similarly, the casein micelles in buffalo milk (50) and donkey milk (51) are considered to be less hydrated and more mineralized than those in cattle milk.

It should be highlighted that there is a high degree of variation in the results that have been reported for the casein micelle characteristics within the same species, which may be due to differences in the analytical methods used. In addition, differences in breeds, genetic variants, and phosphorylation sites of the caseins may also add to the variation in the characteristics of the casein micelles within and across species (13).

Milk Fat Composition

Compared with milk fat from other species (especially ruminants), human milk fat contains lower proportions of saturated fatty acids, higher proportions of monounsaturated

TABLE 3 | Casein characteristics of milk from different mammalian species[#].

Properties	Ruminants						Non-ruminants		Human
	Cattle	Buffalo	Goat	Sheep	Red deer	Camel	Horse	Donkey	
Casein micelle diameter (nm)	150–182	176[A]-180	180–301[B]	180–210	190[C]	380	255	100–200	64–80
Hydration (g H_2O g protein $^{-1}$)	1.92–3.7[D]	1.90[G]	1.43–2.05[F]	1.71–1.93[E]	No data	1.70[I]	No data	∼1.0[H]	No data

[#]*Source: Adapted and modified from Claeys et al. (19).*

Values derived from other references are represented by uppercase case letters: A, Roy et al. (29); B, Nguyen et al. (42), Pierre et al. (43), and Pierre et al. (44); C, Roy et al. (29); D, Dalgleish (45), Wang et al. (46), and Dewan et al. (47); E, Pellegrini et al. (48); F, Remeuf et al. (49); G, Ahmad et al. (50); H, Luo et al. (51); I, Beaucher et al. (52).

fatty acids and polyunsaturated fatty acids, a higher ratio of ω-6 to ω-3 fatty acids, and higher levels of cholesterol (**Table 4**). In general, horse and donkey milk contain lower proportions of saturated fatty acids and higher proportions of polyunsaturated fatty acids than ruminant milks. In contrast, ruminant milks contain higher proportions of monounsaturated fatty acids, a higher ratio of ω-6 to ω-3 fatty acids, and a higher cholesterol content than horse and donkey milk (**Table 4**). The conjugated linoleic acid content is similar in human and ruminant milks but is lower in non-ruminant milks (**Table 4**).

Sheep and goat milk fats are known to be rich in short chain (responsible for the distinct flavor of these milks) and medium chain triacylglycerols (TAGs); similarly, buffalo milk fat contains higher proportions of medium chain TAGs than cattle milk, which has high proportions of long chain TAGs (57–60). In contrast, camel milk contains a higher proportion of long chain fatty acids and a lower proportion of short chain fatty acids than cattle milk (61). Data for the fat composition of red deer milk are scarce, but this milk is considered to contain 5–10% fewer unsaturated fatty acids and higher proportions of shorter chain and saturated fatty acids than cattle milk (21). These differences may contribute to the different digestion behaviors of the milk fat from different species, as short or medium chain TAGs are considered to be more efficiently hydrolyzed by lipases (62, 63).

Free long chain saturated fatty acids, such as palmitic acid (C16:0), are not considered to be efficiently absorbed in the body as they form insoluble fatty soaps with calcium in the small intestine (64, 65). In this context, the TAG structure is considered to play a key role. Most of the long chain palmitic acid (C16:0) present in human milk (>70%) is located in the sn-2 position of the TAG structure; this position is considered to be suitable for the digestion and absorption of this fatty acid as well as other nutrients (18, 62, 66). German and Dillard (64) stated that the location of saturated fatty acids, such as long chain palmitic acid on the sn-2 position of TAGs, makes both the sn-1 and the sn-3 position fatty acids easily hydrolyzable by pancreatic lipases into free fatty acids, and produces sn-2 monoacylglycerols, which are easily absorbed in the small intestine; this also makes the milk calcium completely available and absorbable. Donkey milk has the closest proportion of palmitic acid located at the sn-2 position (i.e., 54%) to that of human milk (74%) (**Table 4**). Thus, the modification of the TAG structure in milk from other species may help to deliver better milk fat digestion profiles; this could be an area of future interest.

Milk Fat Globule Size

The fat in the milk of all species is present as small spherical droplets, called globules, the diameter of which ranges from 0.2 to 15 μm (67). The size of these fat globules varies among milk from different species; goat, sheep, camel, and equine (horse and donkey) milk have higher proportions of smaller size fat globules compared to cattle milk (**Table 5**). The differences in the sizes of the fat globules of milk from different species may influence the digestion of their fat differently (18, 19). The TAG core of the fat globules from all species is surrounded, protected, and stabilized by a phospholipid trilayer (along with specific membrane proteins) called the milk fat globule membrane (MFGM) (68, 69). The MFGM is unique to milk and its structure is considered to be similar in all milks, although the proportions of different proteins in the MFGM may differ among different species (70).

In general, the differences in the characteristics of the casein micelles and the fat globules among different milks are considered to play important roles in influencing their coagulation behavior and nutrient delivery during digestion, which is discussed in the section on milk digestion.

HYPOALLERGENIC POTENTIAL OF NON-CATTLE MILKS

More than 20 proteins in cattle milk are known to cause allergic reactions; of these, the casein fractions (especially α_{s2}-, α_{s1}-, and κ-caseins as well as, to some extent, β-casein), lactoferrin, serum albumin, and β-lactoglobulin are considered to be the most common cattle milk allergens (71–73).

There is increasing interest with respect to the suitability of non-cattle milks as a hypoallergenic option to cattle milk (74). A few studies have reported that horse milk (75), donkey milk (76, 77), camel milk (78, 79), and water buffalo milk (80) may be potential alternatives in cases of moderate allergenicity to cattle milk in children; however, this needs to be further investigated because weak cross-reactivity of non-cattle milk proteins with cattle milk proteins has been reported (81–83). Jenkins et al. (71) conducted a comprehensive study on the cross-reactivity of human and non-human milk proteins and found that the

TABLE 4 | Fatty acid profile (% of total fatty acids) and cholesterol content of milk from different mammalian species[#].

	Ruminants						Non-ruminants		Human
Fatty acids	**Cattle**	**Buffalo**	**Goat**	**Sheep**	**Red deer**	**Camel**	**Horse**	**Donkey**	
SFA (%)	55.7–72.8	62.1–74	59.9–73.7	57.5–74.6	No data	47–69.9	37.5–55.8	46.7–67.7	39.4–45
MUFA (%)	22.7–30.3	24.0–29.4	21.8–35.9	23.0–39.1	No data	28.1–31.1	18.9–36.2	15.31–35.0	33.2–45.1
PUFA (%)	2.4–6.3	2.3–3.9	2.6–5.6	2.5–7.3	No data	1.8–11.1	12.8 –51.3	14.17–30.5	8.1–19.1
ω-6: ω-3 fatty acids ratio	2.1–3.7	No data	4	1.0–3.8	No data	No data	0.3–3.5	0.9–6.1	7.4–8.1
CLA (%)	0.2–2.4	0.4–1	0.3–1.2	0.6–1.1	No data	0.4–1	0.02–0.1	No data	0.2–1.1
Cholesterol (mg/100 mL milk)	13.1–31.4	4–18.0	10.7–18.1	14–29.0	No data	31.3–37.1	5.0–8.8	No data	14–20
% of C16:0 at sn-2	38	37	36	29	No data	No data	No data	54	74

[#]*Source: Adapted and modified from Claeys et al. (19) and Crowley et al. (13).*
SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; CLA, conjugated linoleic acid, C16:0, palmitic acid on the sn-2 position of milk TAG.

TABLE 5 | Fat globule size of milk from different mammalian species[#].

	Ruminants						Nonruminants		Human
Property	**Cattle**	**Buffalo**	**Goat**	**Sheep**	**Red deer**	**Camel**	**Horse**	**Donkey**	
Fat globule diameter (μm)	2.8–4.6	4.1–8.7	2.6–3.7[A]	3.0–4.6	2.7–6.6[A]	3.0	2–3	1–10	4

[#]*Source: Adapted and modified from Claeys et al. (19) and Crowley et al. (13).*
Values derived from other references are represented by uppercase case letters: A, Roy et al. (29).

degree of allergenicity of a non-human milk protein is related to its extent of similarity with its human homologs. They found that, compared with cattle, goat, and sheep milk proteins, camel and horse milk proteins (i.e., α_{s1}- and β-caseins) are more homologous to their human milk counterparts, which may be a reason for their weak cross-reactivity or less allergenic nature compared with other non-cattle milks.

Infante et al. (84) reported that 25% of patients had a negative immunological test for adverse reactions to goat milk proteins; thus, goat milk cannot be considered to be a suitable alternative in cases of cattle milk allergy. Similarly, there is also strong evidence of allergenicity or positive cross-reactivities of goat, sheep, deer, and buffalo milk with cattle milk (83, 85–87). In addition, reports concerning selective allergy to goat and sheep milk proteins, but not to cattle milk proteins, are also available (88, 89). Bevilacqua et al. (90) found that goat milk with lower proportions of αs_1-casein (and higher amounts of αs_2-casein) was significantly less allergenic in guinea pigs than goat milk with high α_{s1}-casein content (and low α_{s2}-casein content); thus, different proportions of milk proteins may also play a key role in controlling milk protein allergy.

Overall, the scientific evidence indicates that there is little basis for promoting non-cattle milk or milk proteins as an alternative to cattle milk for people suffering from cattle (or cow) milk allergy.

MILK DIGESTION

Indispensable Role of the Gastric Phase in Milk Digestion

It is well-accepted that milk is a source of nutritionally balanced and highly digestible proteins (91, 92). Previous studies have reported that the gastric emptying rates of two major fractions of milk protein (i.e., casein and whey protein) differ markedly; this has led to the concept of "slow" digested caseins and "fast" digested whey proteins (93–98).

The digestion of milk by the stomach enzymes (mainly pepsin and, to some extent, gastric lipases) in the presence of hydrochloric acid is considered to be the first key step, which is followed by further digestion in the small intestine by intestinal proteases and lipases (99, 100). Some human infants may have chymosin like enzyme along with pepsin, which disappears from the gastric fluid by day 11 after birth (101). Chymosin and pepsin belong to the same group of aspartic proteinases that uses aspartic acid residues in their active center (102). Both the enzymes can preferentially hydrolyze the Phe105–Met106 bond of κ-casein, except that pepsin also exhibits unspecific proteolytic activity toward bonds with Trp, Tyr, Leu or Val residues, and thus have higher proteolytic activity relative to its milk clotting activity than chymosin (102–104). As the site of action of both chymosin and pepsin is the same, the mechanism of action of chymosin and pepsin is expected to be similar in relation to milk clotting. Chymosin is most stable in the pH range 5.3–6.3, but loses its activity rapidly under acidic conditions, i.e., below pH 3–4, as well as at high alkaline pH values, i.e., above pH 9.8 (105). Pepsin has maximum proteolytic activity at pH 2, with an optimum pH range of 2–5, and has activity in the pH range pH 5.5–7.5. Pepsin is irreversibly inactivated at pHs above 7.5 (106).

The protein hydrolysis sites of pepsin are different from those of the intestinal proteases (mainly trypsin and chymotrypsin). Pepsin acts preferentially on κ-casein on the casein micelles, leading to the coagulation of the casein fraction of milk proteins under acidic conditions, whereas the whey protein fraction remains soluble (107). Thus, the early role played by the stomach

in milk digestion is an essential step in regulating the rate of digestion of the milk proteins in the gastrointestinal tract (108). In this respect, it is of great importance to understand the digestive dynamics and coagulation behavior of milk during gastric digestion, as milk coagulation can influence the delivery rates of proteins, fats, and associated milk constituents.

Evidence of Milk Coagulation

Human milk is known to form very soft and fragile curds in the infant stomach. Mason (109) investigated the changes in pH and the extent of protein hydrolysis in the stomach contents collected using a gastric tube at different time intervals from 25 healthy newborn infants (full-term, aged between 5 and 13 days). He reported the presence of casein curds in the stomach contents collected after 30 min of breastfeeding. He also reported that there was negligible protein hydrolysis in these samples. Similarly, recently, de Oliveira et al. (110) studied the gastric digestion of raw and pasteurized human milk in tube-fed preterm infants. The microstructural analysis in their study showed that human milk formed very soft and fragile protein aggregates in the infant's stomach.

Piglets and growing pigs have been regarded as a suitable animal model for human digestion research (111–113). Bottle-fed piglets have been used to study the digestion of human milk and infant formulas (114–116). Some evidence of clot (or curd) formation by cattle milk in pigs or piglets has been reported in the literature. Washburn and Jones (117) reported that cattle skim milk formed a tough or hard clot, whereas cattle whole milk formed a more friable and mellow curd in the stomach of baby pigs (28–35 days old), and that, the higher the fat content, the softer was the curd that formed. Braude et al. (118) found that the caseins from homogenized cattle milk clotted in the stomach of the 28-day-old pig after 15–30 min of feeding, whereas the "whey" fraction of the milk remained soluble and passed rapidly into the small intestine. Similarly, Decuypere et al. (119) reported the formation of firm casein clots in the stomachs of early weaned pigs (10–29 days of age) fed dry cattle-milk-based food; their gastric chyme had a long retention time and a low buffering capacity and stimulated more gastrin release, compared with the gastric contents of suckling piglets fed pig milk. They believed that these differences were due to the firm casein clot formed by a dry cattle-milk-based food in early weaned pigs in comparison with the soft casein aggregate formed from pig milk in suckling piglets.

Clotting Characteristics of Human Milk and Cattle Milk and Its Implications

Cattle milk is known to form firm curds (or clots) in the stomach, in comparison with human milk.

Nakai and Li-Chan (108) studied the coagulation characteristics of human and cattle milk using an *in vitro* acid precipitation test at 37°C, in which they added 0.2% acidic pepsin solution to 100 mL each of cattle milk and human milk at a flow rate of 15 mL/h. They found that human milk formed much finer protein aggregates (or clots) than cattle milk and reported that this could be the possible reason for the shorter gastric emptying time for human milk.

The differences in the structures of human and cattle milk curds could be related to the differences in their fat and protein compositions. The protein (casein)-to-fat ratio of human milk is very low (**Tables 1**, **2**) compared with that of cattle milk (as well as of other non-cattle milks), which is likely to be a factor that is responsible for its soft (or fragile) curd formation. In addition, the higher β-casein-to-α_s-casein ratio of human milk has been associated with the fine and loose curd formed by human milk in an infant stomach. Lichan and Nakai (120) performed an *in vitro* coagulation study with untreated cattle milk casein, rennin-modified cattle milk casein, and human milk casein. The rennin-modified cattle milk casein was a β-casein-rich cattle milk (similar to β-casein-rich human milk) that was produced by selectively eliminating the α_{s1}-casein fraction from cattle milk by a process involving rennet action. Upon acidification of the different casein solutions to pH 2 or pH 4, Lichan and Nakai (120) observed that the hardness of the clot formed from these different casein solutions decreased in the order: cattle milk casein > rennin-modified cattle milk casein (rich in β-casein) > human casein. In another study, Lichan and Nakai (121) also reported that moderate or partial dephosphorylation of cattle milk casein using different phosphatases (calf intestinal alkaline phosphatase and potato acid phosphatase) at pH 4 resulted in the acid-coagulating properties of these modified cattle milk casein solutions being similar to those of human milk as well as in a greater rate of proteolysis compared with the firm clots of untreated cattle milk casein. However, all these studies were *in vitro* physicochemical studies, and further studies in *in vitro* or *in vivo* digestion models need to be conducted to validate such findings.

Blakeborough et al. (122) studied the digestion of human milk, cattle milk, and reconstituted baby formula (based on full cream dry cattle milk powder) using 14-day-old piglets; cattle milk or baby formula formed firm solid curds, whereas human milk formed a very liquid-like coagulum (little solid material) in the piglet's upper gastrointestinal tract. They also determined the bioavailability of zinc (Zn) from these milk systems; they found that, for cattle milk (as well as baby formula), ∼55–72 and ∼60–66% of the Zn was retained in the curds present in the gastric chyme and the intestinal digesta, respectively, whereas, for human milk, ∼43 and 7% of the Zn was retained in the curds present in the gastric chyme and the intestinal digesta, respectively. They suggested that these differences in the distribution and bioaccessibility of Zn in the gastrointestinal tract of piglets fed human milk or cattle milk may have been due to the differences found in the consistency of the casein curds formed by the different milks.

Digestion of Milk From Different Species

Protein Digestion

The lower protein content, lower casein-to-whey-protein ratio, and higher β-casein-to-α_s-casein ratio of human milk compared with milk from other species have been related to its soft curdling properties *in vitro* as well as *in vivo*, as described earlier. Although none of the non-human milks match the composition of human milk, horse, and donkey milk are known to form very weak or fragile gels (or curds or flocs) when acidified or treated with

rennet (123–125) and thus are expected to form soft or fragile curds in the stomach, in comparison with cattle milk, because of their lower casein content. Similarly, some of the ruminant milks, such as goat and camel milk (126–130), are also considered to form soft curds in the stomach when acidified or treated with rennet (or pepsin), because of their lower casein content or larger casein micelle size compared with cattle milk, even though they contain comparatively higher proportions of caseins than equine and human milk. However, no direct comparative *in vitro* or *in vivo* digestion studies between cattle and non-cattle milks, focusing on their curd formation characteristics in the stomach, have been reported to date. There are only a few comparative *in vitro* digestion studies on cattle and non-cattle milks, focusing on their protein or fat digestion.

Jasińska (131) compared the degrees of hydrolysis by pepsin and trypsin of micellar caseins obtained from cattle, human, goat, and horse skim milk; the peptic hydrolysis rates of the micellar caseins from cattle, human, goat, and horse milks were 23–42 (differed for different breeds of cattle), 80, 65, and 43%, respectively. The tryptic hydrolysis rates of the micellar caseins from cattle, human, goat, and horse milk were 76–90, 100, 96, and 92%, respectively. The higher susceptibility of human and goat milk was believed to be due to the smaller micellar aggregates and the presence of higher proportions of β-casein in their micellar structures, when compared with cattle milk (which had higher proportions of α_{s1}-casein).

Recently, Hodgkinson et al. (132) studied the *in vitro* static gastric digestion of cattle and goat whole milk (at pH 3.0) and reported that, after both 20 and 60 min of digestion, goat milk caseins were digested faster than cattle milk caseins (based on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) image analysis), possibly because of the relatively soft or fragile coagulum formed by goat milk. Tagliazucchi et al. (133) also studied the *in vitro* static gastrointestinal digestion of cattle, goat, sheep, and camel skim milk (as per the INFOGEST protocol) and reported that the extent of free amino groups generated during the gastric digestion was higher for goat, sheep, and camel milk proteins, indicating that the proteins in these non-cattle milks were hydrolyzed faster than cattle milk proteins during the gastric step. However, after the intestinal step, they reported that only the goat milk proteins were hydrolyzed faster than the milk proteins from the other species, all of which had similar hydrolysis rates. Tagliazucchi et al. (134) and Rutella et al. (135) reported similar findings in their previous studies, i.e., that the degree of hydrolysis of goat skim milk proteins during the gastric and intestinal steps was much higher than that of cattle skim milk proteins. The authors stated that the higher degree of hydrolysis of goat milk proteins observed in all studies was probably due to the higher susceptibility of goat milk proteins to pepsin.

Maathuis et al. (136) investigated the comparative protein digestibilities and qualities (based on bioaccessible nitrogen and amino acids) of human milk, cattle-milk-based infant formula, and goat-milk-based infant formula using the tiny-TIM model (a dynamic *in vitro* infant gastrointestinal model). They found that the protein digestibilities and qualities of all diets were similar; however, the rates of protein digestion were slower during the first 60 min of digestion for the cattle-milk-based formula than for the human milk and the goat-milk-based formula. They hypothesized that the differences in the clotting characteristics of different milks would have led to differences in their gastric emptying, as they found that the curds formed from the cattle-milk-based formula were retained for a longer duration in the gastric compartment of tiny-TIM compared with those from the human milk and the goat-milk-based infant formula. Similarly, Ye et al. (32) investigated the *in vitro* dynamic gastric digestion of goat- and cattle-milk-based formulas in a mini version of the human gastric simulator (HGS), simulating infant gastric digestion. The authors found that the goat-milk-based infant formula formed smaller protein aggregates in the mini-HGS, leading to faster hydrolysis of its proteins compared with those from the cattle milk formula. Based on the above-mentioned studies it appears that the differences in the structures of the curds formed from milk of different species during gastric digestion may be a key factor that is responsible for their different digestion behaviors.

In contrast, Almaas et al. (137) did not find any differences in the digestion of caseins and α-lactalbumin from cattle and goat skim milk (with high and low αs_1-casein content) after static gastrointestinal digestion using human gastric juice (HGJ) and human duodenal juice (HDJ). They also did not find any differences between goat milk with high and low αs_1-casein content after digestion with HGJ and HDJ. However, they observed (using SDS-PAGE image analysis) that goat milk β-lactoglobulin was rapidly digested during both gastric digestion and intestinal digestion, compared with cattle milk β-lactoglobulin. El-Zahar et al. (138) studied the hydrolysis of isolated β-lactoglobulin from sheep and cattle milk by porcine pepsin and found that β-lactoglobulin from sheep milk was hydrolyzed faster because of its slightly different tertiary structure and higher surface hydrophobicity. As β-lactoglobulin is considered to be one of the major allergens (as it is absent in human milk), the higher degree of hydrolysis by pepsin of the β-lactoglobulin in goat and sheep milk may be a possible reason that these non-cattle milks are better tolerated by some people than cattle milk.

Vithana et al. (23) studied the comparative *in vitro* gastrointestinal digestion of raw cattle and deer skim milk. They found that, after gastric digestion, nearly 49 and 27% of the deer and cattle milk caseins remained undigested (SDS-PAGE image analysis), respectively, whereas, after intestinal digestion, the caseins from both species were completely digested. This, indicated that, during the gastrointestinal digestion, deer milk caseins were digested at a faster rate than cattle milk caseins. We hypothesize that the higher amounts of caseins retained in the gastric phase for deer skim milk may have been due to the higher protein content (as well as casein content) of the deer milk used in their study, indicating that the inherent composition of milk also has a key role to play during gastric digestion. Vithana et al. (23) also found that α-lactalbumin was hydrolyzed faster in deer milk than in cattle milk. However, β-lactoglobulin from both species was found to be resistant to both gastric and duodenal digestion.

In contrast to the above studies, some studies have reported no differences or faster hydrolysis of cattle milk proteins than

of goat milk proteins. For instance, Inglingstad et al. (139) reported (based on SDS-PAGE image analysis) that 69 and 82% of the caseins remained undigested after hydrolysis by HGJ of cattle and goat skim milks respectively; however, after further treatment with HDJ, almost all of the caseins from the milk of both species were digested. They found that the β-lactoglobulin and α-lactalbumin from both species were highly resistant to HGJ and that, after hydrolysis with HDJ, ∼64% of the β-lactoglobulin from both species remained undigested and 91 and 65% of the α-lactalbumin from the cattle and goat skim milk respectively, remained undigested. Mros et al. (140) reported no differences in the protein digestion of cattle, goat, and sheep skim milk following hydrolysis by pepsin and pancreatin.

Similarly, Milan et al. (141) reported that whole goat-protein fortified milk, compared to whole cow-protein fortified milk, was digested and metabolized similarly (despite the differences in their inherent nutrient composition) in young adults (aged 18–28 years). However, they dissolved paracetamol in fortified milk drinks before giving it to the participants for consumption (plasma paracetamol levels were used as a marker for gastric emptying). It has to be noted that depending on the type of paracetamol used, it may have a buffering action during the gastric digestion in the stomach (142) and thus, careful consideration needs to be made while conducting human digestion studies to draw any firm conclusions.

Vaisman et al. (143) investigated the gastric emptying times in humans of camel and cattle milk using a scintigraphic technique and reported that the poor coagulation properties of camel milk (as observed during acid or rennet coagulation) did not provide any comparative advantage over cattle milk in terms of gastric emptying. It should be noted that the soft or fragile curd formed from non-cattle milks (such as camel, goat, horse, and donkey milk) during acid or rennet coagulation provides only an indication of how these non-cattle milks may behave in the human stomach during gastric digestion. The gastric digestion process is a complex and dynamic phenomenon, and in-depth comparative *in vitro* and *in vivo* studies on cattle and non-cattle milks that simulate the gastric digestion in humans need to be undertaken, to draw any definite conclusions.

Not only protein composition and (or) casein micelle structure, but also different processing temperature and time combinations may induce differences in the curd structure in the stomach, which may influence the rate of delivery of proteins to the small intestine and their subsequent absorption. For instance, Ye et al. (107) studied the dynamic gastric digestion behavior of raw and heated (90°C for 20 min) cattle skim milk using an HGS. The HGS is a dynamic stomach model that is capable of simulating the stomach contraction forces and the flow of gastric fluids that occur *in vivo* (144). Ye et al. (107) found that raw milk formed a "closely knitted" tight clot, whereas heated milk formed fine and loose protein aggregates (**Figure 2**), leading to slow hydrolysis of caseins from raw milk, compared with heated milk. This was because, in raw milk, only the caseins were involved in clot formation, whereas, in heated milk, both the caseins and denatured whey proteins were involved in clot formation (145). Heating at 90°C for 20 min would have led to complex formation between fully denatured whey proteins and caseins via sulfhydryl groups and disulfide linkages (**Figure 3**),

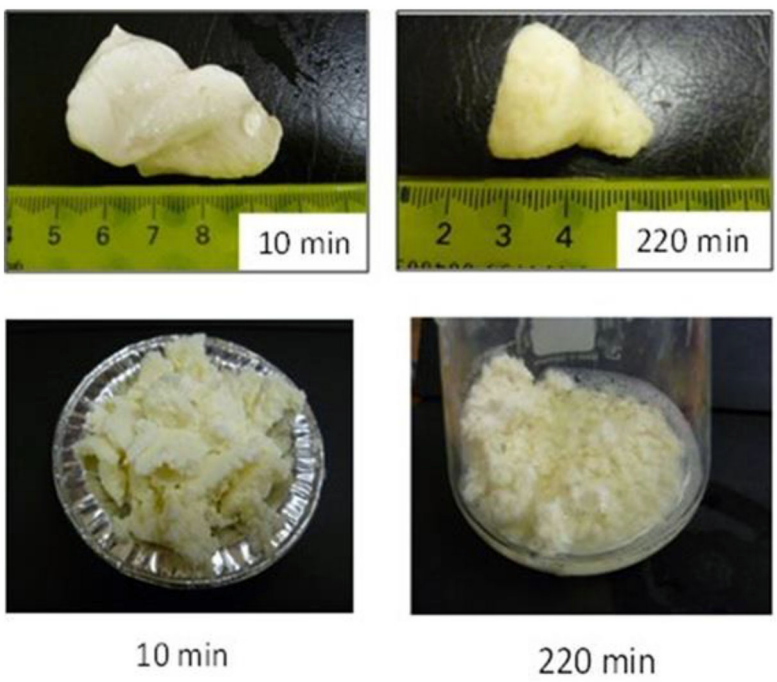

FIGURE 2 | Images of clots formed during the gastric digestion of 200 g of unheated (top row) and heated (bottom row) cattle skim milk at different digestion times. *Source:* Adapted from Ye et al. (107).

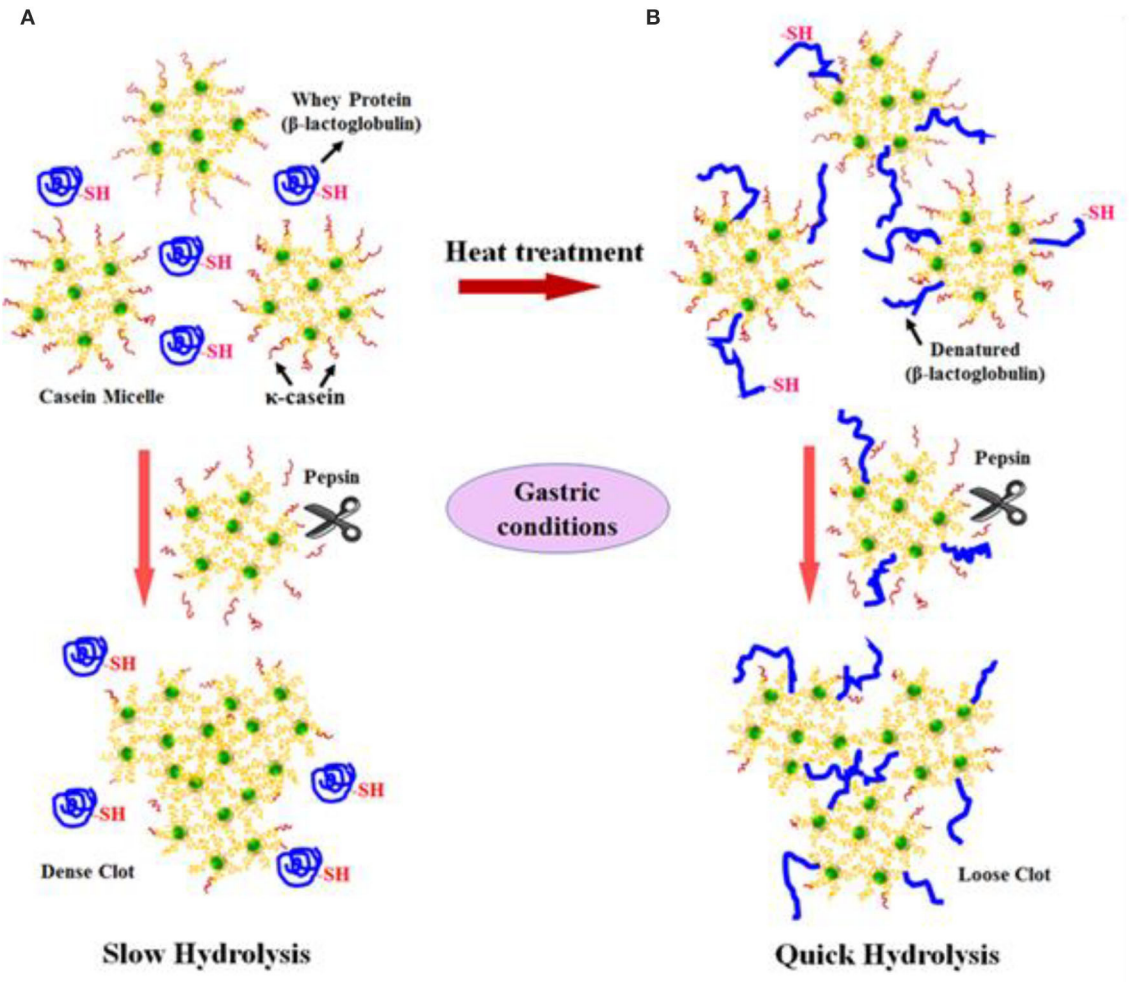

FIGURE 3 | Schematic diagram of the possible mechanism of events during the formation of protein curds from **(A)** raw milk (unheated) and **(B)** heated milk during gastric digestion. *Source:* Adapted from Ye et al. (145).

hindering the formation of a firm clot (146, 147). Kaufmann (148) reported that ultrahigh-temperature-treated (UHT) milk led to the formation of soft coagulates in the mini-pigs stomach, leading to higher levels of amino acids and urea in their blood serum compared to that of pasteurized and raw milk, which formed stronger coagulum. Thus, these differences in gastric restructuring induced by heating are expected to be a key possible reason for higher postprandial utilization of dietary nitrogen from defatted UHT milk (140°C for 5 s) compared to defatted pasteurized milk (72°C for 20 s) as well as defatted microfiltered milk in humans (149).

Doan (150) published a comprehensive review based on studies on the gastric digestion of processed (boiled, evaporated, or acidified) and raw cattle milk in the early 1900s, and reported that boiled, evaporated, or acidified milk were emptied rapidly from the human stomach because of the finer or softer curd that formed. It was suggested that the modification of raw cattle milk using different processing conditions may be a potential option in the development of dairy-based baby foods or beverages with properties similar to those of human milk.

To date, no studies on the impact of different heating or processing conditions on the digestion behaviors of non-cattle milks have been reported in the literature. It should be noted that the commercial processing or technological conditions needed for non-cattle milks may be different from those needed for cattle milk. In addition, the impact of different processing conditions on the digestion behaviors of non-cattle milks may be different from that on cattle milk because of the differences in their composition and structures.

The Influence of the Protein Network on Fat Digestion—The Whole Milk Matrix

During the gastric digestion of whole milk, the fat globules are known to be physically entrapped within the protein clot that is formed. Thus, the nature or structure of the protein network formed will influence the rate of release and the digestion of fat

by gastrointestinal lipases (145, 151–153). Previous studies have shown that the nature or structure of the protein network formed is, in turn, dependent on the protein composition (casein-to-whey-protein ratio), the protein-to-fat ratio, and the impact of different processing conditions (99, 154). For instance, Mulet-Cabero et al. (154) studied the *in vitro* gastrointestinal digestion of model systems based on different casein-to-whey-protein ratios using a semidynamic gastric model, and reported that the viscosity or firmness of the coagulum formed increased as the casein-to-whey-protein ratio increased in the model protein systems, leading to slower gastric emptying, and slower digestion and absorption of nutrients. They also found that the addition of increasing amounts of fat to the casein-rich protein models produced more fragmented clots with a significant decrease in their firmness. This, indicates that the presence of fat hindered the aggregation of proteins, which may, in turn, influence the digestion rates of nutrients.

Ye et al. (151) studied the gastric digestion of raw (unheated) and heated (90°C for 20 min) cattle whole milk and reported that the release of fat globules was dependent on the disintegration characteristics of the protein clot and that the release of fat globules was higher from the finer aggregates of protein clots formed from the heated whole milk than from the firm clots formed from the raw whole milk (**Figure 4**). Similarly, Ye et al. (145) studied the comparative *in vitro* and *in vivo* (in rats) gastric digestions of raw (nonhomogenized), pasteurized (homogenized), and UHT (homogenized) cattle whole milk, and reported that the UHT milk had faster rates of protein hydrolysis as well as release of fat globules during gastric digestion, compared with the raw and pasteurized milk; the differences were attributed to the smaller or fragmented protein aggregates formed from the UHT milk proteins in comparison with the aggregates from the other milks.

In another gastric digestion study, Ye et al. (152) reported that the release of fat globules was relatively higher in homogenized milk (20/5 MPa (primary/secondary pressure), 20°C) as well as heated, homogenized milk (20/5 MPa, 20°C + 90°C for 20 min) because of the fine and crumbled structure of the coagulum formed in these milks compared with the firm coagulum formed from raw cattle whole milk (**Figure 5**). Similar results have been reported by Mulet-Cabero et al. (153) for processed cattle whole milks.

The coalescence of fat globules entrapped within the protein network as well as those present in the liquid phase of the gastric chyme has also been reported in the above-mentioned studies (145, 152, 153), which is expected to be due to the hydrolysis of the proteins present at the surface of the milk fat globule (present naturally in the MFGM or adsorbed proteins because of processing treatments), leading to destabilization of the fat globules and coalescence.

As the milk from different species are known to vary in fat content, protein-to-fat ratio, fat globule size, and structure, there may be differences in the consistency of the coagulum formed from milk of different species during gastric digestion, which may impact their overall digestion behavior differently.

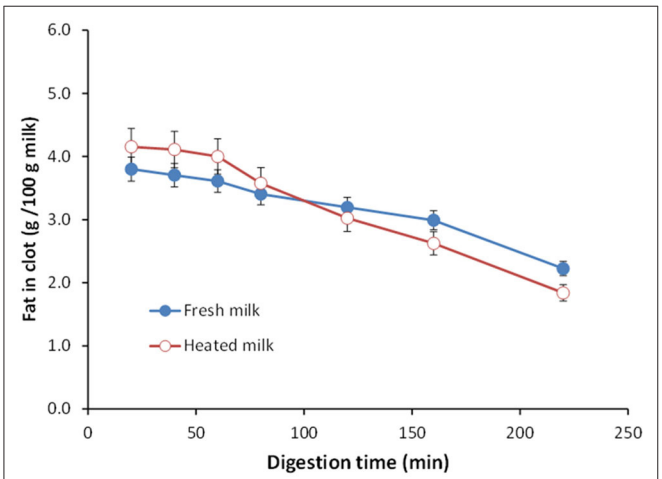

FIGURE 4 | Changes in the fat content (g/100 g milk) in clots obtained from (•) unheated (raw) and (○) heated cattle whole milk during gastric digestion. *Source:* Adapted from Ye et al. (151).

Gastrointestinal Digestion of Fat

Little information is available on the gastric digestion of milk fat, irrespective of species. Lipolysis during the gastric phase was previously considered to be of less relevance during the overall digestion process as gastric lipolysis accounts for only 10–25% of the overall lipid digestion in adults (155). Therefore, most of the studies reported in the literature on fat digestion have focused mainly on intestinal digestion. However, it is now widely suggested that gastric lipases should be incorporated in *in vitro* digestion studies as their preliminary role may facilitate further breakdown of lipids by intestinal lipases (155). Also, in contrast to adults, gastric lipases play a significant role in infants because of their high postprandial gastric pH (156).

It is hypothesized that, the smaller the fat globule size, the higher will be the fat digestibility, because the higher surface area of smaller fat globules will help in rapid digestion via gastrointestinal lipases (13, 18, 19, 157). Meena et al. (158) investigated the digestion of milk fat by pancreatic lipase in standardized raw cattle, buffalo, camel, and goat whole milk. The authors found that the amount of free fatty acids released followed the order: goat ∼ camel > cattle > buffalo. The higher digestibility of goat and camel whole milk was believed to be due to the small size of their fat globules, as the fat globule sizes of the different milks were in the order: buffalo (3.9–7.7 μm) > cattle (1.6–4.9 μm) > goat (1.1–3.9 μm) ∼ camel (1.1–2.1 μm). In addition to the fat globule size, the outer surface of the fat globule and its structure (i.e., the fat globule interface) have a crucial role to play in the digestion of fats. For example, the presence of adsorbed proteins (caused by processing such as heating and homogenization) at the interface of fat globules may result in providing easy access of lipases to the TAG core of the fat globules and thus in influencing the digestion of milk fat (157).

Some studies have also shown the influence of differences in the milk fat composition among different milks on their digestibility. For instance, Alférez et al. (159) studied the fat digestibility and metabolism in feces samples of male albino rats

FIGURE 5 | Images of clots formed during the gastric digestion of raw (unheated), homogenized, and heated homogenized cattle whole milk during 20 and 160 min of gastric digestion. *Source:* Adapted from Ye et al. (152).

that were fed diets containing lyophilized goat and cattle whole milk. They found that, compared with the rats on the cattle-milk-based diet, the digestive utilization of fat was higher, and the levels of cholesterol were lower, in the rats on the goat-milk-based diet. The authors believed that the differences may have been due to the greater amounts of medium chain TAGs and the smaller fat globule sizes of the goat milk fat compared with the cattle milk fat used in their study. Similarly, Teng et al. (160) studied the *in vitro* gastric digestion of raw (non-homogenized) and homogenized cattle and sheep milk, and reported that the TAGs from both raw and homogenized sheep milk were digested by rabbit gastric lipases more rapidly than those from cattle milk; this was due to the presence of higher levels of medium chain fatty acids at the sn-1 or sn-3 position of the TAG structure in sheep milk compared with cattle milk, emphasizing that the structural characteristics of TAGs have an important role to play in their gastric digestion.

Overall, the digestibilities of the protein and fat in milk are likely to be functions of the unique compositions, protein profiles, fat compositions, casein micelle and fat globule structures, interfacial properties, mineral distributions, and physicochemical properties, all of which are likely to be affected to different degrees by the processing conditions, depending on the animal species. Although there are very few studies on the impact of the processing conditions and the milk composition of non-cattle milks in the literature, the principles of cattle milk protein coagulation and its impact on fat digestion are expected to also be applicable to non-cattle milks. However, as cattle and non-cattle milks vary in protein composition (proportion of different proteins) as well as protein-to-fat ratio, it is likely that there will be differences in the structure and consistency of the protein curd (or clot) formed from different milks, which may lead to further differences in the release of fat globules from the clot matrix of different milks. It should also be noted that the gastric and intestinal digestion conditions of infants (as well as the elderly) are different from those of adults in terms of acid secretions and enzyme (proteases and lipases) activities (155, 156, 161). Thus, relevant dynamic *in vitro* models need to be used to study the digestion of milks in different age groups, and *in vitro* results need to be ultimately corroborated based on *in vivo* observations.

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

As non-cattle milk and milk products are highly regarded as a potential source of human nutrition, they can be utilized

to develop specialized dairy products for people in all age groups. Non-cattle milks are of great interest to people as well as industries, because of their perceived better nutritional properties compared with cattle milk. However, most of these presumptions are based on anecdotal reports and only little scientific research has been conducted to understand the nutritional and physicochemical properties of non-cattle milks. One widely perceived notion is the formation of soft curds in the human stomach for some non-cattle milks (such as goat, camel, horse, and donkey milk). Because of this, these milks are considered to be better digested and tolerated by people of different age groups. However, to date, no direct scientific studies have been reported and there is a knowledge gap. As cattle and non-cattle milks vary in composition and structure of the casein micelles and fat globules, they are likely to behave differently in the gastrointestinal tract, possibly affecting the kinetics of digestion and the bioavailability of nutrients. Because of differences in milk composition and the structure of the casein micelles (or fat globules), there may be differences in the curds formed by the milk of each species in the stomach, which may further affect the delivery rates of macronutrients further down the gastrointestinal tract. Furthermore, different commercial processing conditions such as pasteurization or UHT (or other heat treatments) may influence the digestion behaviors of non-cattle milks differently. Thus, in-depth scientific studies need to be conducted to understand the impact of compositional as well as structural differences in milk from different species (in their natural form as well as processed forms) on their dynamic digestion behaviors, especially focusing on their differences in curd formation as well as their disintegration properties in the stomach. Such studies will often involve *in vitro* digestion models, which where possible should be dynamic and sophisticated enough to at least include the effects of key variables known to influence food digestion. Further, the physiological relevance of such phenomena needs to be investigated in animal and human studies focusing on different age groups or people in need of targeted personalized nutrition (such as infants, the elderly, athletes or malnourished people).

AUTHOR CONTRIBUTIONS

DR prepared the original draft and edited the manuscript. HS critiqued and edited the original draft of the manuscript. AY and PM critically reviewed the manuscript. All authors listed have made a substantial, direct, and intellectual contribution to the conception and design of the manuscript and read and approved the final manuscript for publication.

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Milk Bank Pooling Practices Impact Concentrations and Variability of Bioactive Components of Donor Human Milk

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Background: Donor human milk (DHM) bank practices, such as pasteurization and pooling according to postpartum age of milk donations and number of donors included in a pool may impact the resulting concentration of bioactive components of DHM.

Aims: We determined the impact of Holder pasteurization, postpartum milk age, and pool donor number (number of donors included in a pool) on resulting concentrations of total immunoglobulin A (IgA; which provides immune protection to the recipient infant) and insulin (an important hormone for gut maturation).We also documented inter-relationships between these bioactive components and macronutrients in DHM pools.

Methods: Pre and post-pasteurization aliquots of 128 DHM samples were obtained from the Rocky Mountain Children's Foundation Mother's Milk Bank (a member of the Human Milk Banking Association of North America, HMBANA). Macronutrients were measured via mid-infrared spectroscopy. Total IgA was measured via customized immunoassay in skim milk and insulin was measured via chemiluminescent immunoassay.

Results: Mean post-pasteurization total IgA concentration was 0.23 ± 0.10 (range: 0.04–0.65) mg/mL a 17.9% decrease due to pasteurization ($n = 126$). Mean post-pasteurization DHM insulin concentration was 7.0 ± 4.6 (range: 3–40) μU/mL, a decrease of 13.6% due to pasteurization ($n = 128$). The average DHM pool postpartum milk age was not associated with total IgA or insulin concentrations, but pool donor number was associated with bioactive components. Pools with only one donor had lower total IgA and lower insulin concentrations than pools with at least 2 donors ($p < 0.05$). Increasing the number of donors in a pool decreased the variability in total IgA and insulin concentrations ($p < 0.04$).

Conclusion: Increasing the number of donors included in DHM pools may help optimize bioactive components in DHM received by premature infants. These results help inform milk banking practices to decrease compositional variability in produced DHM pools.

Keywords: donor human milk, insulin, IgA, milk bank, breastfeeding

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INTRODUCTION

The American Academy of Pediatrics recommends pasteurized donor human milk (DHM) as the best option to feed a premature infant when mother's own milk is unavailable (1). The Human Milk Banking Association of North America (HMBANA) supplies millions of ounces of pasteurized DHM annually to recipient infants (2). The majority of this milk is provided to premature infants in the Neonatal Intensive Care Units (NICU), due to the protective effects of DHM against necrotizing enterocolitis (3, 4).

Human milk composition is dynamic, changing over the course of a feed, over the course of a day, and over the course of lactation. For these reasons, DHM banks routinely pool milk donations from multiple donors in order to limit extreme variation. Criteria used to select individual donations for pooling are not standardized and vary among milk banks. Currently, milk postpartum age (age of the infant at the time milk was expressed) is not typically taken into account when pooling individual donations. Furthermore, milk banks each have different acceptance criteria for milk age.

Upon pooling, DHM is aliquoted and pasteurized using Holder Pasteurization (62.5°C for 30 min and rapid cooling). At this stage, bottles are frozen and distributed. It is well-documented that Holder Pasteurization reduces the concentration of various bioactive components of HM. However, the degree to which individual bioactive components are degraded is widely variable, and not consistently reported in the literature (5).

A biologically active component of HM is immunoglobulin A (**IgA**) (6). HM total IgA is one of the components that provides breastfed infants passive protection against infections during infancy (7, 8). Secretory IgA (a dimer of two IgA attached to a secretory component) is the predominant type of immunoglobulin A present in HM and provides infants with passive immune protection (6, 7). Total IgA concentrations in HM decrease over the first year of lactation (6, 9). Additionally, Holder Pasteurization has been reported to reduce IgA concentrations by 20–62% (5, 9, 10).

HM insulin plays important roles in infant intestinal maturation (11), may improve feed tolerance (11), impact the developing microbiome (12), and has been linked to infant growth patterns and body fat accrual (13). Insulin in HM is predominantly impacted by maternal insulin sensitivity (14), a factor not considered in the donor milk pooling process. Additionally, current data suggest that HM insulin decreases over the first month of lactation (14), and is also significantly decreased by Holder Pasteurization between 13–46% (15, 16).

Because IgA represents a significant component of total HM protein, and because both HM insulin and total fat have been reported to correlate with maternal BMI (17–19), we also tested for correlations between DHM IgA and insulin with DHM macronutrients.

Given the importance of these bioactive components of HM, we aimed to characterize the mean and variability of total IgA and insulin concentrations in HMBANA-produced DHM pools and investigate the impact of Holder Pasteurization and relationships between milk postpartum age and number of donors included in a pool.

MATERIALS AND METHODS

Donor Human Milk Bank Samples

This research received designation as "Not Human Subject Research" by the Colorado Multiple Institution Review Board.

Pre- and post-pasteurization samples from 128 DHM pools were obtained from the Colorado Mothers' Milk Bank (Rocky Mountain Children's Health Foundation, Arvada, CO). This milk bank selects individual donations to pool based on the expiration date of donated milk. Milk donations are considered to "expire" 12 months post expression date. DHM pools are then aliquoted, pasteurized in glass bottles via Holder Pasteurization and frozen for distribution.

Preterm milk pools included only human milk expressed by a mother who delivered at or before 36 weeks gestation, within the first 4 weeks postpartum and up until her infant's corrected age is 40 weeks. Pool donor number was defined as the number of milk donors represented in each pool. Pool postpartum age was defined as the unweighted mean of the postpartum ages of all the donations included in any given pool as previously described (17).

Milk Analyses—Total IgA

Total IgA concentrations of pre- and post-pasteurization samples were measured using an adapted version of a commercially available immunoassay (Bethyl Laboratories, Montgomery, Texas). A flat bottom 96 well Maxisorp plate was coated with a 1:100 dilution of anti-human IgA (Bethyl Lab A80–102A) in 0.05M carbonate-bicarbonate: 100 uL per well, overnight at 4°C. The following day, 200 uL of 1% bovine serum albumin (BSA) in PBS was added to each well to block the plate for 30 min at room temperature. Skim milk was generated by spinning samples at 10,000 g for 10 min at 4°C. Skim milk samples were diluted 1:5000 in 1% BSA in PBS and then incubated on the plate for 2 h at room temperature. Assay controls were prepared from a serial dilution of control serum (Bethyl Lab RS10–110) in 1% BSA in PBS. Secondary antibody (Bethyl Lab A80–102P) was added at a concentration of 1:100,000 in 1% BSA in PBS and incubated for 1 h at room temperature: 100 uL per well. Following the 1 h incubation time, 50 uL of stop solution (2M H_2SO_4) was added to each well.

The plate was washed after the following: blocking, sample incubation, and secondary antibody incubation using a BioTek Microplate Washer and a PBS-T solution with 0.05% Tween-20 for five cycles of a 300 μL wash. The absorbance was then read at 450 nm and a 4PL standard curve was plotted within 30 min of adding the stop solution. Samples were assayed in duplicate and re-assayed if %CV of duplicates was >25%. The assay average %CV between replicates was <10%.

Milk Analyses—Macronutrients and Insulin

DHM macronutrients (fat, protein, carbohydrate, and caloric density) were formerly assessed using the Miris Human Milk Analyzer (Miris AB, Uppsala Sweden) as previously reported

(17). Insulin concentrations were measured in pre- and post-pasteurization skim milk samples via chemiluminescent immunoassay utilizing an automated immunoassay platform (Beckman Coulter), as previously reported (14).

Statistical Analyses and Calculations

Comparison of milk composition between Preterm vs. Term DHM pools and by pool donor number was conducted using non-parametric tests. For comparison by pool donor number, DHM pools were categorized as consisting of donations from 1, 2, or ≥3 donors. A comparison of milk composition between pre-pasteurized and post-pasteurized samples was conducted using paired *t*-tests.

Simple linear regression was used to determine if the pool Postpartum Age was related to milk composition, and if any individual milk components were inter-correlated. Log transformation was used on any non-linear variables. The normality of these regression's residuals was established to ensure model assumptions were satisfied. The Brown–Forsythe test was performed to test whether variation in log DHM Total IgA and Insulin concentrations differed by pool donor number. Analyses were performed using JMP Pro 14 (SAS, Cary NC).

RESULTS

Characteristics of Pools

Out of the 128 DHM pools, 5 (3.9%) of the pools were "Preterm," and 4 (3.1%) of the pools were "Dairy Free" meaning donors completely omitted dairy products from their diet. Data regarding pool donor number (number of donors in each pool), pool volume, and milk age have been previously reported (17). Of the 128 pools, 55 pools consisted of 1 donor; 62 pools consisted of 2 donors; and 11 pools consisted of ≥3 donors.

Pool Composition–Total IgA

Two of the 128 post-pasteurization samples were inconclusive for total IgA analysis due to high %CV, giving a final sample size for post-pasteurization total IgA analysis of 126. Two of the term post-pasteurization samples were not included in the final data analysis in order to not bias results, as both were outliers over five standard deviations from the IgA mean (1.575 and 1.212 mg/mL). Excluding these data points did not change the nature of reported relationships.

As expected, given the Holder pasteurization process, total IgA concentrations were higher in pre-pasteurized DHM pools. The mean ± SD of total IgA concentrations in pre-pasteurization samples was 0.31 ± 0.14 mg/mL ($n = 128$) vs. 0.23 ± 0.09 mg/mL in post-pasteurization samples ($n = 126$). The total IgA concentrations in post-pasteurization samples decreased on average 0.08 mg/mL. The range in decrease was −0.61 to + 0.19 mg/mL), or 17.9% ($p < 0.001$; **Table 1**).

The DHM total post-pasteurization IgA concentrations were not correlated with pool postpartum milk age. DHM total post-pasteurization IgA concentrations did differ by pool donor number: DHM pools with one donor had a lower total IgA concentration than pools with at least two donors ($p = 0.04$; **Figure 1**). Increasing the number of donors in a pool significantly decreased the variability in resulting total IgA concentrations ($p = 0.027$).

The DHM total post-pasteurization IgA concentrations did not significantly differ between Preterm and Term pools, nor was DHM Total post-pasteurization IgA associated with DHM macronutrient composition.

Pool Composition—Insulin

DHM insulin decreased an average of 12.6% following pasteurization; from 8.1 ± 5.7 μU/mL (range: 3–54 μU/mL) to 7.0 ± 4.6 μU/mL (range: 3–40 μU/mL). The range in decrease was −15 to + 3 μU/mL. DHM post-pasteurization insulin concentrations were not associated with pool milk age.

DHM total post-pasteurization insulin concentrations did differ by pool donor number: DHM pools with one donor had lower insulin concentrations than pools with 2 donors ($p = 0.003$; **Figure 1**). Increasing the number of donors in a pool significantly decreased the variability in resulting total insulin concentrations ($p = 0.034$).

DHM insulin concentrations did not differ between term and preterm pools. The log of DHM pool insulin concentrations was positively associated with DHM fat content ($p < 0.0001$, $R^2 = 0.16$, $n = 128$), and caloric density ($p < 0.0001$, $R^2 = 0.15$, $n = 128$).

DISCUSSION

This study demonstrates a large variability in DHM total IgA and insulin concentrations. Holder pasteurization resulted in significant decreases in both bioactive components. While postpartum milk age was not associated with variation in total IgA and insulin concentrations, pool donor number was associated with significant differences in the mean and total variation of both.

This data corroborates others' findings suggesting that total IgA is affected by the pasteurization process. Other studies reported a decrease in total IgA due to Holder Pasteurization within the range of 20–62% (5, 9, 10), which is higher than we detect here (18%). Previously reported studies simulated milk pooling in a laboratory setting in smaller sample sizes, whereas this study includes a larger sample size and DHM directly from a HMBANA milk bank, which may account for the difference in percent–decrease.

Even though milk IgA decreases over time in the first year of lactation, DHM total IgA was not associated with milk postpartum age. This is in contrast to our previous work showing that zinc concentrations, which also decline over the course of lactation, are inversely correlated with DHM pool postpartum age (17). These zinc data are similar to others' work showing that DHM amino and fatty acid composition is also related to postpartum age (20). However, other studies of macronutrient composition of DHM pools have documented that subject effect (i.e., variability between donors) had a higher impact on macronutrient composition than time effect (i.e., pool postpartum age) after 4 weeks postpartum (21, 22), even though human milk macronutrient composition is known to

TABLE 1 | Total IgA and Insulin Concentrations in Pasteurized Donor Human Milk.

Component	Sample	Mean	Standard deviation	Range	Percent decrease
Total IgA (mg/mL)	Pre-pasteurization	0.31	0.14	0.06–0.84	17.9
	Post-pasteurization[a]	0.23	0.10	0.04–0.62	
Insulin (μU/mL)	Pre-pasteurization	8.1	5.7	3.0–54.0	12.6
	Post-pasteurization	7.0	4.6	3.0–40.0	

[a]*Sample size = 126; Otherwise, sample size = 128.*

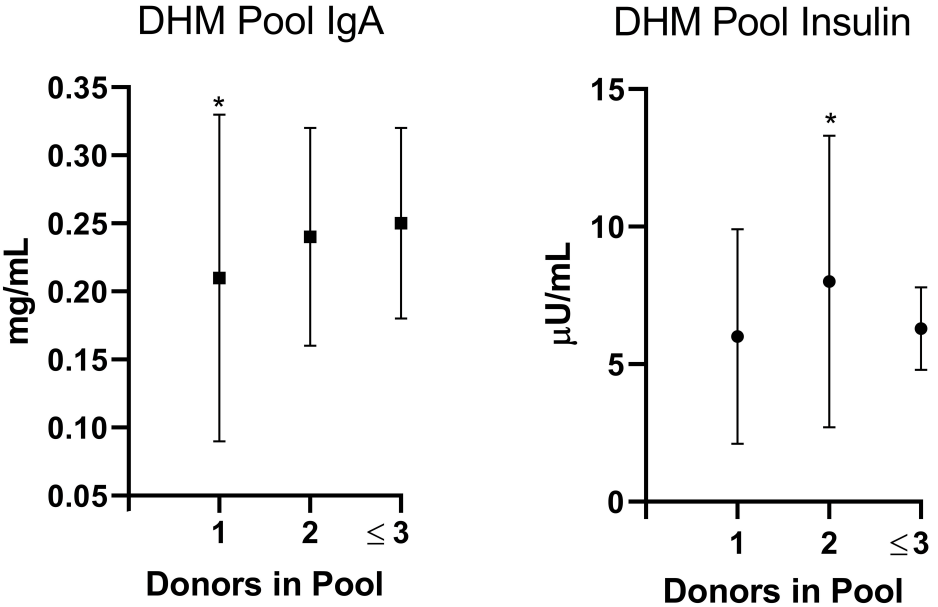

FIGURE 1 | Donor Human Milk (DHM) Immunoglobulin A (IgA, mg/mL) and Insulin (μU/mL) concentrations differ by Pool donor number (the number of donors included in the pool). Pools including one ($n = 54$) vs. two ($n = 61$) vs. ≤3 ($n = 11$) donors have different concentrations of IgA (*$p = 0.04$) and Insulin (*$p = 0.003$), by Wilcoxan test. Furthermore, the variation in concentrations was lowest in DHM pools consisting of ≤3 donors (IgA $p = 0.027$; Insulin $p = 0.034$). Mean ± standard deviation presented.

change over time. These findings reflect our own with total IgA concentrations.

DHM total IgA in postpartum samples did differ by pool donor number with lower concentrations in pool from one donor, and less variability in pools with 3+ donors. This finding validates the HMBANA policy of pooling milk, ideally from three to five donors (23). However, milk banks may occasionally face limited capacity to meet this recommendation based on fluctuations in availability of donations. In a study of over 300 DHM pool samples from 20 milk banks, 55% of studied pools consisted of donations from ≥3 donors (24). This study collected 15 random samples from 20 milk banks, whereas our data represent consecutive DHM pools generated over several different days in one bank which likely contributes to the differences in the amount of DHM pools consisting of ≥3 donors. Given that IgA in DHM may protect infants from infection, any efforts to optimize and standardize its provision to medically fragile infants is worthwhile.

Our data suggest that a recipient infant could be exposed to a wide range in insulin concentrations from one DHM pool to another. The role of insulin in HM is complex and yet to be fully characterized (25). Animal data suggest that milk insulin directly affects intestinal gene expression and maturation (5, 10, 26–29). Data from human infants indicate that milk insulin also contributes to intestinal lactase expression and feed tolerance (30), as well as playing a role in regulation of infant weight gain (31) and body composition (13).

It is noteworthy that we detected an average decrease of 13% in insulin due to Holder pasteurization, with 34% of samples showing no decrease. This reduction is less than the 46% decrease in average insulin reported by Ley et al. (15), but very similar to the 13% reduction in averages reported by Vass et al. (16). Similar to total IgA, DHM insulin was not related to milk pool age, but was related to pool donor number with lower concentrations in pools from one donor, and lower variation in pools with ≥3 donors.

We also detected a correlation between DHM insulin and total fat concentrations (and thus caloric density as well). This relationship may reflect the underlying relationship between maternal BMI and both human milk insulin concentrations

(17), and milk fat, as several studies have reported that women with higher BMI and/or obesity produce milk with higher fat content (18, 19). Currently, maternal phenotype, including BMI, is not taken into account during the pooling process. Given the shortage of milk donations in the US, it does not seem warranted that maternal BMI be considered during pooling.

A strength of our study is usage of pre- and post-pasteurized DHM pools produced by a HMBANA milk bank, as opposed to small batch lab-generated pools. An additional strength of the study is the large sample size, which allows for an accurate estimate of the variation in pool characteristics. Our inability to link DHM pools and the total IgA and insulin concentrations to outcomes of recipient infants, or to characteristics of individual donors may be considered a weakness. Furthermore, these samples were generated from one milk bank where 9% of pools consisted of ≥3 donors over the days of pool production studied, which may not be representative of other milk banks. These findings help inform milk bank practices to optimize the benefit of DHM provided to recipient infants. Additionally, these findings help inform neonatologists about the mean and variability of bioactive components in DHM fed to premature infants.

In conclusion, we have shown that DHM pools yield a wide range in concentrations of two potent bioactive components: total IgA and insulin. The number of donors included in a DHM pool affect both the final concentrations and the variability in total IgA and insulin. As such, there may be a benefit to standardizing the procedures for pool generation across independent milk banks to provide a more uniform "dose" of these bioactives to recipient premature infants. Lastly, these data support the need for both social and cultural initiatives to ensure donor milk banks have enough donation availability to meet pooling goals.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

BY, LB, RH, and NK contributed to the conception and design of the study. LB, RH, BY, and KM collected the data. BY organized the database. BY and KM performed the statistical analysis. BY and KM wrote the first draft of the manuscript. All authors contributed to manuscript revisions, read, and approved the submitted version.

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Dietary Patterns, Body Composition, and Bone Health in New Zealand Postmenopausal Women

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Nutrition affects bone health status. However, analysis of the dietary patterns gives insights into which particular combination of foods may influence nutritional status and bone health. The aim of this study was to explore the associations between dietary patterns, bone mineral density (BMD) and T-scores, and body composition in New Zealand postmenopausal women. This cross-sectional study examined 125 postmenopausal women aged between 54 and 81 years. Body composition, BMD and T-scores were determined using dual-energy X-ray a bsorptiometry (DXA). Diet composition was assessed using a validated food frequency questionnaire (FFQ) composed of 108 food items, from which 34 food groups were created. Dietary patterns were identified by principal component analysis. The bone and body composition data including skeletal sites T-scores, waist circumference, BMI and body fat percentage were regressed onto the dietary patterns. Four dietary patterns were identified; the milk and milk-rich beverages dietary pattern, the dessert, cheese and red meat dietary pattern, the fruit-rich, biscuit and crackers dietary pattern and the oily fish, sports drink and seafood-rich dietary pattern. The milk and milk-rich beverages dietary pattern was significantly positively associated with spine T-score ($r = 0.247$, $P = 0.008$), and not whole-body BMD ($r = 0.182$, $P = 0.051$). The oily fish, sports drink and seafood-rich dietary pattern was marginally negatively associated with waist circumference ($r = -0.157$, $P = 0.094$) and body mass index ($r = -0.163$, $P = 0.081$) and significantly associated with body fat percentage ($r = -0.247$, $P = 0.008$). Binary logistic regression indicated that intake of the milk and milk-rich beverages dietary pattern reduced the occurrence of osteoporosis [adjusted odds ratio OR (95% CI): 0.589 (0.353, 0.982)]. A dietary pattern characterized by a high factor loading of milk and milk-rich beverages was positively associated with whole-body BMD and spine T-score, while the oily fish, sports drink, seafood-rich dietary pattern was inversely associated with total body fat percentage. Consumption of milk, even with coffee showed a positive association with bone health among postmenopausal women. Further longitudinal intervention studies is warranted to confirm effects of dietary patterns on skeletal body sites such as hip and femoral neck T-scores.

Keywords: foods, dietary pattern, bone health, body composition, postmenopausal women, New Zealand

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INTRODUCTION

Maintaining bone health in older age is a public health issue/challenge especially for populations at risk such as postmenopausal women. Extensive research studies have reported the relationship between dietary factors and its effect on bone health (1–3). Consumption of high amount of fruits and vegetables (1), milk, dairy products and green tea (4) as well as a Mediterranean-style diet (5) has been associated with lower risk of osteoporosis. Likewise, consumption of low-fat plant-based dietary intervention has been linked to weight loss (6) while meat-based diets were linked to higher blood pressure (7).

The risk is heightened for postmenopausal women with estrogen deficiency related to both the early and late stages of osteoporosis (8). A modifiable mechanism of reducing the effect of menopause on older women is the consumption of a nutrient dense dietary pattern. Dietary patterns rich in nutrients such as calcium, phosphorus, protein and vitamin D have been documented to have a beneficial role for bone mass (9).

Specific diets play a significant role in determining nutritional status and health outcomes, it is therefore imperative to study what patterns of diet affect or contribute to an individual's body composition and bone health status. The consumption of a nutrient dense dietary pattern has been recognized to reduce the effects of menopause in older women. Few studies have investigated the relationship between dietary patterns and body composition (10, 11), no study in New Zealand has investigated the relationship between dietary patterns and BMD. Nutrient dense dietary pattern consumption has been reported to be associated with reduced effects of menopause in older women. Previously, we investigated the association between nutrient patterns of this cohort and their bone parameters using the 3-day diet diary and the nutrient data. We notably showed that a nutrient pattern high in the intake of vitamin E, α-tocopherol and omega 6 fatty acids may be detrimental for bone health in postmenopausal women (12). Nevertheless, we investigated the dietary patterns using the FFQ and food intake data to identify the type of foods that are significant for bone health.

In addition, there are limited studies that have explored the relationships between dietary patterns and nutritional status in New Zealand (13, 14). However, in order to better understand the relationship between dietary patterns and bone health across population in different geographical locations, data from different populations of postmenopausal women are important. Therefore, to support the search for an "ideal" diet, studies from various communities are warranted to enable generalization. To our knowledge no study has previously analyzed the relationship between dietary patterns and bone health in New Zealand postmenopausal women.

We consequently investigated the associations between dietary patterns, bone health parameters and body composition in New Zealand postmenopausal women. The aim of the study was to assess the relationships between dietary patterns and bone health evaluated by BMD site T-scores and nutritional status evaluated by body composition, BMI and waist to hip ratio in postmenopausal women.

MATERIALS AND METHODS

Study Design

This study recruited 127 postmenopausal women aged between 54 and 81 years to participate in the cross-sectional clinical study; "Bugs'n'Bones." The study took place in the Human Nutrition Research Unit located on Massey University Palmerston North campus from June to December 2017. We excluded two participants post recruitment, one was due to the consumption of a ketogenic diet and the other due to health conditions. G*Power software version 3.0.10 was used to calculate the sample size with a 90% power and an alpha of 5%.

Participants were recruited on Massey University campus by advertisement. The advertisement was also placed in a local newspaper (Whanganui Chronicle). In addition, we used a recruitment agency; Trial Facts (https://trialfacts.com/) for further recruitment. The inclusion criteria included healthy women with at least 5 years post menopause. Exclusion criteria were any presence of systemic disease such as diabetes and liver diseases, food intolerances affecting the gut, smokers and excessive intake of alcohol. Participants with any significant weight loss or weight gain (i.e., more than 5%) within the past year were also excluded. All participants were free living and apparently healthy.

All participants provided written informed consent prior to the commencement of datat collection. This study has been registered with the Australian New Zealand Clinical Trials Registry (ANZCTR) with the number ACTRN12617000802303 and was carried out in accordance with the recommendations of Massey University Human Ethics Committee Guidelines, Massey University Human Ethics Committee: Southern A, Application 17/17.

Anthropometric and Body Composition Measurements of the Participants

Body weight of participants using the Detecto 437 eye-level weigh beam physician scale was measured to the nearest 0.1 kg. Standing height using a stadiometer was measured to the nearest 0.1 cm with participants wearing light clothes and no shoes on. Body mass index (BMI) was then calculated as weight in kg divided by height in meters squared. Waist circumference was measured by using a non-stretchable measuring tape to the nearest 0.1 cm.

Body composition measurements were measured and analyzed using the Hologic QDR series Discovery A, Bone densitometry system [Dual energy X-ray Absorptiometry (DXA)]. BMD was measured at the femoral neck (FN), lumbar spine (LS) (L1–L4), and total hip. The DXA machine was calibrated every morning for all the measurements and at the end of each day.

The reproducibility of the coefficient of variation (CV) for the DXA ranged between 0.34 and 0.70% for all measured sites. The reported lumbar spine BMD values were calculated as means of four measured values from L1–L4. The Apex System Software version 4.5.3 was used for analyzing the DXA scans. The osteoporosis classification was defined as a T score ≤ 2.5 and osteopenia as T score between -1.0 and -2.5 according

to the WHO criteria (15) and were both named "OP." The healthy classification was defined as a T-score −1.0 or greater and named "H."

Dietary Intake Assessment and Dietary Pattern Identification

Participants' diets were assessed with a validated semi-quantitative FFQ from the Nutrition Department, Massey University, New Zealand. Diet composition was assessed using a validated food frequency questionnaire (FFQ) composed of 108 food items, from which 34 food groups were created. The FFQ was used to collect information on participants' frequency of food intake and beverage intake. Portion size, food and beverage intake were entered into the Foodworks version 9 Xyris software which was used to analyze the participants' diet data and nutrient analyses of intake were calculated.

Principal component analysis (PCA) is an acceptable exploratory factor analysis for linking a set of variables into smaller dimensions, while varimax rotation enables an orthogonal (uncorrelated) factors that are interpretable. Validated FFQs are a gold standard method of collecting information on quantity and frequency of foods consumed retrospectively (16). Dietary assessment tools are therefore necessary for identifying patterns of an individual's diet in relation to any health issues associated.

PCA was used to identify four dietary patterns using 34 food groups collated from a 108-item semi-quantitative FFQ. The four dietary patterns were chosen based on the eigenvalues greater or equal to 2.0. A total 28.1% variance was explained from dietary pattern 1 (8.2%), dietary pattern 2 (7.8%), dietary pattern 3 (6.3%) and dietary pattern 4 (5.9%). A table of the food groups and items is attached (**Supplementary Table 1**). The analysis was performed for 125 study participants in order to reflect their dietary patterns. Varimax orthogonal rotation with Kaiser normalization was performed to reduce correlations between factors and increase interpretability of the results. Kaiser-Meyer-Olkin measure of sampling adequacy was 0.5 while Bartlett's test of sphericity was significant (<0.001).

Data Analyses

IBM SPSS version 25 (IBM Company, Armonk, NY, USA) was used in this study for all statistical analyses. The outcome variables used were BMD of whole body and the regional skeletal sites T-score and the body composition parameters. The values of all variables for the whole body and regional sites were presented as mean ± standard error. The dietary patterns consisted of the food intakes such as milks (all types of milk), fruits, vegetables, yogurt/cream, white fish, red meat, coffee etc. The dietary patterns were obtained from the dimension reduction of 34 food items. We conducted binary logistic regression of the osteoporotic status (H or OP) against the dietary patterns adjusting for age, BMI and activity energy expenditure (AEE). The outcomes, i.e., bone health and body composition parameters, were regressed onto the dietary patterns generated using the "enter" method in linear regression. All *p*-values were reported significant at 0.05 or less.

TABLE 1 | Characteristics of participants.

Parameters	Healthy (60)	Osteopenic/ osteoporotic (65)	*P*-value
	Mean ± SE	Mean ± SE	
Age (years)	62.32 ± 0.64	62.91 ± 0.49	0.463
Waist circumference (cm)	84.73 ± 1.41	77.24 ± 1.17	<0.001
BMI (kg/m²)	27.87 ± 0.55	24.88 ± 0.44	<0.001
Spine BMD	1.07 ± 0.10	0.82 ± 0.08	<0.001
Spine T-score	0.23 ± 0.91	−2.02 ± 0.09	<0.001
Hip BMD	0.92 ± 0.01	0.79 ± 0.01	<0.001
Hip T-score	−0.18 ± 0.90	−1.21 ± 0.09	<0.001
Femoral neck BMD	0.76 ± 0.09	0.66 ± 0.07	<0.001
Femoral neck T-score	−0.74 ± 0.11	−1.69 ± 0.08	<0.001
Total energy intake (kJ)	9269 ± 446	7608 ± 257	0.001
AEE (kJ/min)	4724 ± 1856	1516 ± 680	0.097

BMI, body mass index; BMD, bone mineral density; AEE, activity energy expenditure; SE, standard error.

RESULTS

The results from **Table 1** illustrate that the postmenopausal women had an average age of 62 years. The mean BMI was 27.9 kg/m² for the healthy and 24.9 kg/m² for the women with osteoporosis. The **Table 1** also shows the BMD and T-scores for the skeletal sites differentiating healthy from osteopenic/osteoporosis based on the spine T-score classification. Based on these criteria, 60 women were classified as healthy and 65 were classified as osteopenic/osteoporotic women.

Table 2 shows the dietary pattern factor loadings of the 34 food groups identified from the FFQ. Four dietary patterns have been named based on the factor scores high loading as shown in **Table 3**. The dietary pattern 1 consisted of a high factor loading of milk and milk-rich beverages. The dietary pattern 2 is high in desserts, cheese and red meat. Dietary pattern 3 comprised of high factor loadings of fruit, biscuits and crackers, potato and bread making it a carbohydrate-rich pattern. The dietary pattern 4 was composed of high factor loadings of oily fish (whole fish with fish bone), sport drinks and seafood.

In **Table 3**, a binary logistic regression of the association of the osteoporotic status against the dietary patterns was conducted. The result showed that dietary pattern 1 namely milk and milk-rich beverages dietary pattern significantly reduced the odds of being osteoporotic amongst the women. The odds of being osteoporotic was almost halved with dietary pattern 1 [OR (95% CI): 0.589 (0.353, 0.982)].

The milk and milk-rich beverages dietary pattern was significantly positively associated with spine T-score ($r = 0.247$, $P = 0.008$) but not with the whole-body BMD ($r = 0.182$, $P = 0.051$). However, this DP was not significant for hip and femoral neck T-score although a relatively high β-coefficient was recorded

TABLE 2 | Factor loadings of dietary patterns.

Foods	Dietary patterns			
	1	2	3	4
Milks	**0.600**			
Vegetables	**–0.534**		0.286	−0.147
Processed meat	**–0.478**	0.188	−0.158	−0.110
Coffee	**0.462**	0.102	−0.148	−0.145
Malt and chocolate beverages (non-alcoholic)	**0.450**		0.211	
Soup	**–0.430**	−0.287	0.414	
White fish	**–0.416**		0.277	0.123
Seafood	**–0.394**	**0.367**		**0.313**
Confectionery	**0.304**	0.257		−0.105
Pizzas/burgers	0.295	0.161		−0.152
Spirits	0.249	0.227		
White meat	−0.233			−0.172
Dessert	0.129	**0.718**	0.194	
Cheese	−0.174	**0.697**	0.107	
Red meat	−0.106	**0.491**		−0.286
Sauces/dressings	−0.261	**0.425**		−0.252
Carbonated drinks		**0.387**		
Juice	0.116	**0.360**		0.183
Wine		**0.304**	−0.216	−0.129
Fruit	**0.302**		**0.467**	
Biscuits and crackers	−0.102		**0.467**	−0.104
Spreads			**0.444**	0.179
Crisps/nuts	−0.116	−0.109	**0.440**	
Tin/dry fruit			**0.401**	
Tea	**–0.312**	0.120	**0.400**	
Yogurts and cream	0.243	**0.359**	**0.373**	0.274
Rice/pasta		0.235	**0.343**	0.135
Bread			**0.338**	−0.200
Cereal/porridge	0.191		0.213	
Cake and Pie			−0.202	−0.188
Oily fish (Sardine and Tuna)		−0.181		**0.763**
Sport drinks	0.120	0.103		**0.742**
Potato	**0.333**	−0.128	**0.320**	**–0.413**
Beer				0.228
Variance explained (%)	8.2	7.8	6.3	5.9

Extraction method: principal component analysis.
Rotation method: varimax with Kaiser normalization.
Rotation converged in nine iterations.
Bold value represents, Factor scores ≥ 0.300.

TABLE 3 | Logistic regression results of the association between osteoporotic status and dietary patterns after adjustment for age, BMI and activity energy expenditure.

Dietary patterns	Odds ratio (95% C.I)	*P*-value
Dietary pattern 1	0.589 (0.353, 0.982)	0.042
Dietary pattern 2	0.710 (0.444, 1.136)	0.153
Dietary pattern 3	0.655 (0.411, 1.046)	0.077
Dietary pattern 4	0.858 (0.460, 1.600)	0.629

Dietary pattern 1 = Milk and milk-rich beverages dietary pattern, Dietary pattern 2 = Dessert, cheese, and red meat dietary pattern, Dietary pattern 3 = Fruit-rich, biscuit, and crackers dietary pattern, Dietary pattern 4 = Oily fish, sports drink, and seafood-rich dietary pattern.

TABLE 4 | Results of multiple linear regression of the dietary patterns (score values) and bone status[a].

Parameters		β-coefficient 95%	Confidence interval *P*-value
Spine T-score			
Dietary pattern 1	0.247	0.094, 0.609	0.008
Dietary pattern 2	0.129	−0.071, 0.427	0.159
Dietary pattern 3	0.082	−0.138, 0.368	0.368
Dietary pattern 4	0.034	−0.202, 0.296	0.707
Femoral neck T-score			
Dietary pattern 1	0.158	−0.028, 0.316	0.099
Dietary pattern 2	0.016	−0.151, 0.178	0.869
Dietary pattern 3	0.034	–.134, 0.194	0.719
Dietary pattern 4	−0.091	−0.354, 0.123	0.339
Hip T-score			
Dietary pattern 1	0.148	−0.038, 0.334	0.117
Dietary pattern 2	0.007	−0.173, 0.187	0.937
Dietary pattern 3	−0.015	−0.198, 0.168	0.873
Dietary pattern 4	−0.082	−0.260, 0.100	0.382
Whole body BMD			
Dietary pattern 1	0.182	−0.020, 0.020	0.051
Dietary pattern 2	0.009	0.009, 0.049	0.925
Dietary pattern 3	0.091	−0.033, 0.007	0.325
Dietary pattern 4	−0.038	−0.031, 0.010	0.681

[a]*Crude model.*
Dietary pattern 1 = Milk and milk-rich beverages dietary pattern, Dietary pattern 2 = Dessert, cheese, and red meat dietary pattern, Dietary pattern 3 = Fruit-rich, biscuit, and crackers dietary pattern, Dietary pattern 4 = Oily fish, sports drink and seafood-rich dietary pattern.

for femoral neck ($r = 0.158$, $P = 0.099$) and hip T-score ($r = 0.148$, $P = 0.117$) signifying a positive association (**Table 4**).

Table 5 shows the multiple linear regression of the dietary patterns against the body composition parameters. Of note is the significant negative relationship between the oily fish, sports drinks, seafood-rich dietary pattern and the total fat percentage. High negative correlations were also observed between waist circumference, BMI and dietary pattern 4 (Oily fish, sports drink and seafood-rich dietary pattern). For the waist circumference ($r = -0.157$, $P = 0.094$) and BMI ($r = -0.163$, $P = 0.081$) and dietary pattern 4, a negative association was also observed but was not significant.

DISCUSSION

The objective of the present research was to investigate the relationship between the dietary patterns generated from the FFQ provided by the women and their bone health status and body composition. The women were 5 years past menopause. The findings of this study showed that the high loading factor of milk

TABLE 5 | Results of multiple linear regression of the dietary patterns (score values) and body composition[a].

Parameters		β-coefficient 95%	Confidence interval *P*-value
Waist circumference			
Dietary pattern 1	−0.081	−3.034, 1.184	0.387
Dietary pattern 2	0.123	−0.676, 3.408	0.188
Dietary pattern 3	−0.043	−2.561, 1.586	0.642
Dietary pattern 4	−0.157	−3.780, 0.302	0.094
BMI			
Dietary pattern 1	0.032	−0.660, 0.937	0.731
Dietary pattern 2	0.100	−0.362, 1.235	0.281
Dietary pattern 3	−0.032	−0.938, 0.659	0.731
Dietary pattern 4	−0.163	−1.507, 0.090	0.081
Total fat percentage			
Dietary pattern 1	0.013	−1.142, 1.314	0.890
Dietary pattern 2	0.017	−1.116, 1.340	0.857
Dietary pattern 3	−0.034	−1.461, 0.995	0.708
Dietary pattern 4	−0.247	−2.901, −0.445	0.008

[a]*Crude model.*

Dietary pattern 1 = Milk and milk-rich beverages dietary pattern, Dietary pattern 2 = Dessert, cheese and red meat dietary pattern, Dietary pattern 3 = Fruit-rich, biscuit and crackers dietary pattern, Dietary pattern 4 = Oily fish, sports drink and seafood-rich dietary pattern.

and milk-rich beverages dietary pattern was positively correlated with spine T-score and whole-body BMD for the women. Binary logistic regression analysis also showed a reduction in the odds of being osteoporotic for this dietary pattern. The dietary pattern reports a high loading of milks, milk-rich beverages such as coffee. This may probably be associated to the established coffee culture in New Zealand (17). This milk and milk-rich beverages dietary pattern also had a moderate loading of fruit and yogurt and cream.

The results of this study also highlighted that a dietary pattern characterized by high factor loadings of oily fish (with fish bone), sports drink and seafood and white fish was negatively associated with total body fat percentage and marginally for waist circumference and BMI. The intake of oily whole fish with bones such as sardine and tuna as well as seafood intake was negatively associated with waist circumference, BMI and total body fat percentage. It could be noted that the intake of sports drink which may signify a moderate-vigorous activity, was also negatively related to these body composition parameters.

Although the results of this study cannot be directly compared with that of other studies due to the differences in the protocols such as the number of food group classifications and food records, the dietary patterns generated are similar to those reported previously. The finding of a positive association between dairy and fruit, and bone health was in accordance with other studies that have reported a positive relationship between dairy and fruit dietary pattern and bone health (2–4). The milk and milk-rich beverages dietary pattern in this study was similar to the dairy and fruit dietary pattern obtained by Shin and Joung (2013) from the Korean postmenopausal women (18). Similarly, this dietary pattern was comparable to the "healthy" dietary pattern obtained from Scottish postmenopausal women high in fruit, cheese, yogurt/cream (4). Of note is the reiteration that dairy foods are important for bone health.

Calcium is important for bone metabolism and is a crucial component of the bone matrix (19, 20). Milk and dairy are an important source of calcium while dairy in combination with fruit intake has been reported as a good source of iron, vitamin A, C, K, thiamin, riboflavin, niacin, and magnesium (21–24) linked to bone formation and bone health status mainly in post-menopausal women. The milk and milk-rich beverages dietary pattern would be of great benefit for bone health in post-menopausal women if improved simultaneously with vitamin D status (25). In many countries, milk is supplemented with vitamin D.

The findings of this study also suggest that a dietary pattern with a high loading of oily fish (with fish bone), sports drink, seafood, and white fish was negatively correlated with body composition most especially for total body fat percentage. The oily fish (with fish bone), sports drink and seafood dietary pattern which is similar to the Mediterranean diet was negatively correlated with total body fat percentage. The Mediterranean diet has been reported to be inversely associated with body composition (26, 27). However, sports drink featuring in this dietary pattern maybe as a result of the means by which the women replenish their electrolytes level during physical activity. The physically active women build lean body mass which has been previously positively linked to BMD (28).

Coffee is a marker of high energy and as such body composition should be taken into account for bone health related studies. Moderate coffee intake ($\leq$400 mg/day of caffeine) appears to be potentially beneficial for metabolic health status (29). On the other hand, coffee can also be considered as a dairy (milk) marker which could have been part of the dietary pattern simply because milk is most often consumed with coffee.

Based on this research work and our previously published work on the relationship between nutrient pattern and bone health (12), our recommendation of an "ideal" diet for postmenopausal women would include intake of milk/dairy, whole grain, nuts and seeds, fruit and protein foods with less saturated fat. Quite unexpectedly, the dietary pattern 1 in this study was low in vegetables. The Mediterranean-style diet rich in the consumption of vegetables, fruit, wholegrain, fish, olive oil, and dairy products may be beneficial and is recommended (27).

The strengths of this study include the well-established data-driven statistical methodology and its high total variance explained by the four dietary patterns. The limitations of this study include its cross-sectional methodology and small sample size which could prevent causation should be taken into consideration when interpreting the study.

CONCLUSION

The milk and milk-rich beverages dietary pattern was associated with spine T-score and was borderline for whole body BMD.

A diet rich in milk/dairy may therefore decrease the risks of osteoporosis in New Zealand postmenopausal women. Furthermore, this suggests that no matter how milk/dairy is consumed; the impact of this type of dietary pattern on bone health especially in postmenopausal women might be more important than originally thought. Likewise, intake of oily fish (especially whole fish with bone such as sardine) and seafood may be beneficial for bone health in postmenopausal women. The study also revealed that an oily fish, sports drink and seafood dietary pattern was negatively associated with total body fat percentage.

Overall, the findings of this study suggest that there was a positive association between milk, milk-rich beverages and fruit and fish intake and bone health and may therefore decrease the risks of osteoporosis in New Zealand postmenopausal women. A dietary pattern consisting of oily fish, sports drink and seafood may have a positive influence for weight (fat) management in this population. However, further intervention research is warranted to confirm relationships between these dietary patterns and hip, femoral neck and lumbar spine T-scores in postmenopausal women.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Massey University Human Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BI-O carried out the experimental design, collected the data, conducted the statistical analyses, and wrote the first manuscript draft. JC was involved with the experimental design, data collection and supervision. NR was involved with the experimental design and supervision. MK initiated the experimental concept, was involved in the experimental design, data collection and sourced the research funding. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2020.563689/full#supplementary-material

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Time of Lactation and Maternal Fucosyltransferase Genetic Polymorphisms Determine the Variability in Human Milk Oligosaccharides

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Rationale: Human milk oligosaccharides (HMOs) vary among mothers and genetic factors contribute to this variability. We assessed changes in HMO concentrations during the first year of lactation and the relationship with *FUT2* Secretor group and *FUT3* Lewis group defining genetic polymorphisms.

Methods: Milk samples were collected from lactating mothers participating in the LIFE Child cohort in Leipzig, Germany. The concentrations of 24 HMOs in milk samples collected at 3 months ($N = 156$), 6 months ($N = 122$), and 12 months ($N = 28$) were measured using liquid chromatography. Concentrations of HMOs were compared at all time-points and were tested for their associations with *FUT2* and *FUT3* genetic variations by sPLS regression.

Results: *FUT2* SNP rs601338 was found to predominantly define the Secretor status Se-: 11.8% and it was highly correlated with 2′-fucosyllactose (2′FL, $p < 0.001$) and lacto-N-fucosylpentaose-I (LNFP-I, $p < 0.001$). *FUT3* SNPs rs28362459 and rs812936 were found to define Lewis status (Le-: 5.9%) and correlated with lacto-N-fucosylpentaose-II (LNFP-II, $p < 0.001$). A polygenic score predicted the abundance of 2′FL levels within Secretors' milk (adj. $R^2 = 0.58$, $p < 0.001$). Mean concentrations of most of the individual HMOs, as well as the sums of the measured HMOs, the fucosylated HMOs, and the neutral HMOs were lower at 6 and 12 months compared to 3 months ($p < 0.001$).

Conclusions: Secretor and Lewis status defined by specific *FUT2* and *FUT3* SNPs are confirmed to be good proxies for specific individual HMOs and milk group variabilities. The polygenic score developed here is an opportunity for clinicians to predict 2′FL levels in milk of future mothers. These results show opportunities to strengthen our

understanding of factors controlling FUT2 and FUT3 functionality, the temporal changes and variability of HMO composition during lactation and eventually their significance for infant development.

Keywords: breast milk, HMO, FUT2, oligosaccharides, FUT3

INTRODUCTION

Human milk oligosaccharides (HMOs) are unconjugated glycans found in human milk and they are composed of the monosaccharides glucose, galactose, fucose, *N*-acetylglucosamine and *N*-acetylneuraminic acid, the main form of sialic acid in humans. They represent the third most abundant solid component of the human milk (5 to 20 g/L) after lactose and lipids (1–4). More than 200 HMOs have been separated, and around 150 of these have been characterized (5). Although non-digestible oligosaccharides are found in most mammalian milks (6), the oligosaccharide profile in human milk is of unique diversity (7, 8).

In general, the concentration of HMOs decreases during lactation, although only few studies have gone beyond the first 6 months of lactation (9–11). Generally, the sum of quantified HMOs is highest in colostrum secreted by the mammary gland during the first few days after birth and decreases thereafter (1, 12, 13). Some HMOs have a specific concentration trajectory over time (1) with most decreasing and few increasing in concentration suggesting the presence of regulatory mechanisms that control their temporal variation.

Several fucosyltransferases (FUT) are suggested to be involved in HMO synthesis (14, 15). Of these, FUT2 and FUT3 enzymes catalyze the α1,2-, and α1,3/4- transfer, respectively, of a fucose group to the core oligosaccharide structure (16). Genetic variations on *FUT2* and *FUT3* genes can lead to enzyme inactivation resulting from a premature stop of protein synthesis or a production of a truncated protein with very low activity (17, 18). Mothers who carry these inactivating mutations on *FUT2* are referred to as non-Secretors, as they lack the major α-1,2-fucosylated glycans in body secretions like saliva (19). Based on FUT2 enzyme specificity, the presence or absence of HMOs like 2′-fucosyllactose (2′FL) and lacto-N-fucopentaose-I (LNFP-I) in breast milk is generally discussed to be due to genetic polymorphisms in *FUT2* (18, 20). Phenotypic Secretor status determination and enzyme characterization confirmed this assumption (21). Similarly, milk samples derived from mothers with inactive FUT3 due to genetic variations are referred to as Lewis negative, as opposed to Lewis positive when FUT3 is active. Again, based on enzyme specificity, polymorphisms in *FUT3* are expected to be related to the α-1, 3/4-fucosylated HMOs, like lacto-N-fucopentaose II (LNFP-II) (14).

Milk samples can be assigned to one of four milk groups depending on combinations of presence or absence of HMOs containing α-1,2-linked fucose residues (2′FL, LNFP-I, Secretor-specific) and α-1,4-linked fucose residues (LNFP-II, Lewis-specific) with expected presence or absence of the respective FUT2 and FUT3 enzyme activities (22, 23). Following this, milk group 1 corresponds to Secretor and Lewis positive (Se+Le+), milk group 2 corresponds to Non-Secretor and Lewis positive (Se-Le+), milk group 3 corresponds to Secretor and Lewis negative (Se+Le-) and milk group 4 to Non-Secretor and Lewis negative (Se-Le-) profile. Each milk group appears to bear a specific HMO profile with a characteristic trajectory over time defined by the presence of active fucosyltransferases and substrate availability (1). In addition, previous studies have also shown that Secretor status can affect not only the production of specific fucosylated HMOs but also the overall concentration of HMOs with non-Secretors apparently having a significantly lower concentration of total measured HMOs (13, 24).

Most of the *FUT2* and *FUT3* genetic variations are single nucleotide polymorphisms (SNP) characterized by a replacement of a single nucleotide. Some of them lead to a replacement of an amino acid or an early termination of the protein synthesis (functional SNPs).The most studied example is *FUT2* SNP rs601338 known to be the predominant variant leading to the non-Secretor phenotype in the European population (17, 25). It results in a stop codon that leads to premature termination of protein expression and complete abolition of the enzymatic activity (26). There is, however, no current study in the literature systematically investigating the effect of functional *FUT2* and *FUT3* SNPs on the concentration of individual and total measured HMOs in milk.

We sought to assess the impact of *FUT2* and *FUT3* SNPs on concentrations of HMOs in a cohort of 156 lactating women from Leipzig, Germany at 3, 6 and 12 months, aiming to establish a link between individual genetic variations and specific HMO profiles over the first year of lactation. In addition, we aimed to test how genetic variation in *FUT2* and *FUT3* combined can predict milk groups, as well as concentrations of individual HMOs.

MATERIALS AND METHODS

Study Population

LIFE Child is a longitudinal epidemiological childhood cohort study initiated in 2011 in Leipzig, Germany. The study aims

Abbreviations: 2′FL, 2′-O-Fucosyllactose; 3FL, 3-O-Fucosyllactose; 3′GL, 3′-Galactosyllactose; 3'SL, 3′-O-Sialyllactose; 6′GL, 6′-Galactosyllactose; 6'SL, 6′-O-Sialyllactose; AA, Amino acid; A-tetra, A-tetrasaccharide; Chr, Chromosome; Chrpos, Chromosome position; DFLNHa, Difucosyl-lacto-N-hexaose a; DSLNT, Di-Sialyl-lacto-N-tetraose; FUT2, Fucosyltransferase 2; FUT3, Fucosyltransferase 3; GLM, Generalized Linear Model; Hex, Hexose; HexNAc, N-acetylhexosamine; HMO, Human milk oligosaccharides; IV, Intron variant; LD, Linkage disequilibrium; LDFT, Lactodifucotetraose; Le, Lewis; LNDFH-I, Lacto-N-di-fucohexaose I; LNFP-I,-II,-III,-V, Lacto-N-fucopentaose-I,-II,-III,-V; LNH-a,-b, Lacto-N-hexaose-a,-b; LNnDFH, Lacto-N-neodifucosylhexaose; LNnFP, Lacto-N-neofucopentaose; LNnFP-V, Lacto-N-neofucopentaose V; LNnT, Lacto-N-neo-tetraose; LNT, Lacto-N-tetraose; LSTc, Sialyl-lacto-N-tetraose c; LoQ, Level of Quantification; MAF, Minor allele frequency; MFLNH-III, Monofucosyl-lacto-N-hexaose III; Mis, Missense; NS, Not analyzed; sPLS, Sparse Partial Least Square; Se, Secretor; SG, Stop gain; SNP, Single nucleotide polymorphism; Syn, Synonymous.

to follow children from pregnancy into young adulthood and determine risk and resilience factors for healthy development. The study is described in detail elsewhere (27, 28). In the child's first year of life, visits are scheduled at the age of 3, 6, and 12 months of life. Between 2011 and 2015, 156 lactating mothers visited the study center providing 156 milk samples at the 3-months visit, 122 at the 6-months visit, and 28 at the 12-months visit. Mothers were aged between 23 and 42 years at the child's birth. Blood samples from the mothers were collected between 2011 and 2015. DNA was isolated within 48 h after blood withdrawal on the QIAGEN Autopure LS platform using chemistry by Qiagen and Stratec Molecular and DNA samples were stored at −80°C in the LIFE-Biobank until usage.

Sequencing Samples

DNA quantification was performed using the Picogreen (Life Technologies) ultra-sensitive fluorescent nucleic acid stain for quantifying double-stranded DNA on all samples. For a representative subset of samples, DNA integrity was validated with the TapeStation (Agilent) Genomic DNA ScreenTape.

The entire *FUT2* and *FUT3* coding sequences and part of 3′ and 5′ untranslated regions were PCR amplified for 30 cycles using the Kapa HiFi (Roche), starting from 50 ng DNA. PCR primer sequences were ACACACCCACACTATGCCTG (FUT2-Fw), AAGAGAGATGGGTCCTGCTC (FUT2-Re), CCCGGAGCTTTGGTAAGCAG (FUT3-Fw), and GAGGGTTGGCCACAAAGGAC (FUT3-Re). The same melting temperature of 60°C was used for both amplifications. A positive control (DNA from HapMap NA18523) and a No Template Control (water) were included on each PCR plate. The quality and quantity of each *FUT2* and *FUT3* PCR were checked by gel electrophoresis using the LabChip GX Touch (Perkin Elmer).

After purification on Ampure beads (Beckman) at a 1.8X ratio, sequencing libraries were prepared from the amplicons using the Nextera XT kit (Illumina) strictly following manufacturer's recommendations. Libraries were quantified with Picogreen (Life Technologies) and their size pattern validated with Fragment Analyzer (AATI). Sequencing was performed as a paired end 250 cycles run with the MiSeq Reagent Kits v2 (Illumina). The dataset is available at Sequence Read Archive (SRA) under project ID PRJNA643141.

Calling Genetic Variants

Variant calling was performed with the software FreeBayes Garrison and Marth 2012 using default parameters. In order to perform the computation of such a large dataset, a script to parallelise the computation has been implemented and SNP calling has been split by batch of 200bp-long region. The resulting *vcf* files were then post-processed with the *plink* software v1.9 for quality control (QC) purposes and recoding. The quality check was performed in 3 steps. First, samples with more than 5% of missing genotypes were filtered out (–mind 0.05) and 1 sample was removed due to missing genotype data. Then, variants missing in more than 5% of the samples were filtered out (–geno 0.05): 24 variants were removed due to missing genotype data. Finally, variants with minor allele frequency (MAF: the frequency of the rare polymorphism in the population) below 1% computed on cohort data were filtered out (–maf 0.01), 2435 variants were removed due to minor allele threshold. Finally, 23 SNPs and 152 samples passed the filters and the QC.

Determining Secretor, Lewis Status and Milk Groups

The classification of individuals in each Le/Se type was based on the genotype of functional SNPs; for Lewis (Le) type: rs3745635, rs28362459, rs3894326, rs812936 and for Secretor (Se) type: rs601338, rs1047781, and rs200157007, respectively. If the minor allele was found in homozygote form for at least one SNP, individuals were classified according to group definition, meaning Lewis negative (Le–) and non-Secretor (Se–), respectively. Milk groups were defined as previously described: milk group 1 corresponds to Secretor and Lewis positive (Se+Le+), milk group 2 corresponds to Non-Secretor and Lewis positive (Se–Le+), milk group 3 corresponds to Secretor and Lewis negative (Se+Le–) and milk group 4 to Non-Secretor and Lewis negative (Se–Le–).

Determination of HMOs

HMOs were analyzed according to the method of Austin & Benet (29). Quantification of 2′FL, 3FL, 3′SL, 6′SL, A-tetra, LNT, LNnT, and LNFP-I was performed against genuine standards purchased from Elicityl (Crolles, France). All other HMOs were quantified against maltotriose (Sigma, Buchs, Switzerland) as a surrogate standard assuming equimolar response factors.

Comparison of HMO Concentrations

We tested for differences in HMO concentrations by group of maternal genotypes for each SNP determining Secretor and Lewis status as outlined above. We used a Mann-Whitney test for non-matched non-parametric data with significant level threshold set to 0.0001. We performed pairwise comparisons and only retained the significant ones.

We calculated correlation coefficents between the different HMOs based on their concentrations in milk after log transformation by applying Pearson's product moment correlation coefficient and adjusted for multiple comparisons by controlling the false discovery rate.

Dynamics of HMOs Over Time of Lactation

HMOs were combined in to 3 categories "fucosylated," containing all the measured fucosylated HMOs, "sialylated," containing all the measured sialylated HMOs, and "core" containing the core non-fucosylated HMOs LNT, LNnT, LNH, Hex2HexNAc4, as well as 3′GL and 6′GL, although the latter two are not strictly core structures. Dynamic changes for categories of HMOs (core, fucosylated, and sialylated HMOs) were assessed by fitting quantile regression (30) (tau = 0.5) of log concentrations with time-points in months. Confidence intervals for the estimated parameters are based on inversion of a rank test.

Clusters of HMOs and their boundaries were determined with multiscale bootstrap resampling of the correlation values with complete distance and p-value threshold set to 0.05.

Genetic Markers of Milk Groups

In order to select the best SNP to predict the milk group the mother belonged to, we first performed a Sparse Partial Least Square regression (sPLS) on both the concentration of individual HMOs and the SNP matrices. This resulted in a first selection of 14 SNPs among the 24 measured HMOs. Then in a second step, we performed prediction modeling by testing 46 different models splitting the dataset in training and testing datasets. The Multi-Layer Perceptron (MLP) model performed best with highest accuracy of 0.978, representing the proportion of correct predictions to the total number of predictions. Although other models performed similarly, the MLP has been selected for its ease of interpretation compared to the others. Eventually, combinations of the weights in the network (31) were used to estimate the importance of the variables in the model.

Polygenic Prediction Score for 2′FL in Milk of Secretor Mothers

A Generalized Linear Model (GLM) in both directions was used in a stepwise approach to select the individual and combinations of SNPs that best predict 2′FL concentration in breast milk. Each SNP in the model was encoded by 0, 1, or 2 for homozygous major allele, heterozygous and homozygous minor allele, respectively. The selected model was trained on a training dataset 200 repeats of 40-fold cross validation. The evaluation of the model was performed on an independent test dataset. The selected SNPs were included in an algorithm to compute a genetic score. The genetic score was defined as the sum of the alleles for the SNPs selected in the model. Then we regressed the 2′FL concentration with the genetic score on a training set to define the prediction model. Finally, we tested the prediction on an independent test dataset. We showed the genetic score was able to predict the concentration of 2'FL with an adjusted *R*-Square of 0.58.

RESULTS

Concentrations of HMOs During the First Year of Lactation

HMO concentrations have been measured in breast milk during the first year of lactation at 3, 6 and 12 months of infant age. HMOs were grouped as core structures such as LNT, LnNT, fucosylated structures such as 2′FL, 3FL, and sialylated structures such as 3′SL, 6′SL, (**Figure 1**, **Supplementary Table 1**). The summed concentrations of HMOs decreased from the 3rd to the 12th month of lactation, (**Figure 1**, Core: −0.070, $q = 0.005$, Fucosylated: −0.073, $q < 0.001$, Sialylated: −0.122, $q < 0.001$). This remained true for most individual HMOs regardless of the milk group of the mother (**Supplementary Table 2**). However, 3FL concentrations in milk from mothers with Se+/Le+ status significantly increased during the first year of lactation (**Table 1**).

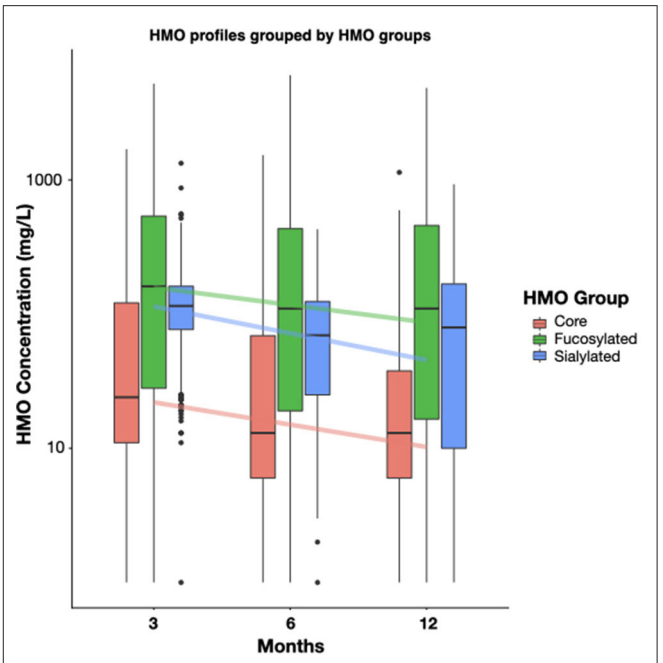

FIGURE 1 | Dynamics of HMO concentration by the 3 main HMO classes, (i) core, (ii) fucosylated, and (iii) sialylated HMOs, between 3 and 12 months of lactation. The core class represents the sum of; LNT, LNnT, Hex4HexNAc2, LNH, 3′GL, 6′GL; The fucosylated class represents the sum of: A-tetra, 2′FL, LDFT, 3FL, LNFP-I, LNFP-II, LNFP-III, LNFP-V, LNnFP-V, LNnDFH,LNDFH-I, DFLNHa, MFLNH-III; The sialylated class represents the sum of: 3′SL, 6′SL, LSTb, LSTc, DSLNT. Boxplots depict median with interquartile ranges and whiskers indicate the minimum and maximum values. For all classes the 3 months to 6 months and the 6 months to 12 months changes were significant by non-parametric *t*-test ($p < 0.05$). Concentrations are shown as mg/L.

Correlations Between HMOs in Our Study Population

We observed significant correlations between several of the HMOs, which could be separated into 5 clusters (**Figure 2**). Cluster 1 included LDFT, LNDFH-I, DFLNHa, 2′FL, and LNFP-I, all of which contain the same structural feature, α-1,2-fucose, dependent on the activity of FUT2. It also contained LNnT and a hexasaccharide with the composition Hex4HexNAc2, which do not have an obvious connection with the other members of the cluster. Cluster 2 included LSTb, DSLNT, LNT, and LNFP-V. These oligosaccharides are all based on LNT as the core structure elongated with 1 or 2 sialic acids or a fucose. Their concentrations tend to be highest when FUT2 and FUT3 are both inactive as seen in previous studies. Cluster 3 contained LNH, MFLNH-III, 6′SL, and LSTc. MFLNH-III is a fucosylated HMO based on LNH as core. 6'SL and LSTc both contain an α-2,6-linked sialic acid residue. However, the connection between the sialylated structures and the LNH-based structures is not obvious. Cluster 4 contained 3′SL, 3′GL, and 6′GL. All three have the common lactose core with an additional galactose or sialic acid residue. Finally, cluster 5 contained LNnFP-V, LNFP-III, LNnDFH, 3FL, and LNFP-II, all

TABLE 1 | Dynamics of individual HMO concentrations during the 3 to-12-month lactation period.

HMO	Milk group	Coefficient	*q*-value < 0.1
2'FL	Se+/Le+	−0.167	0.051
3FL	Se+/Le+	0.204	0.087
6'SL	Se−/Le+	−0.960	0.002
6'SL	Se+/Le−	−0.888	0.059
6'SL	Se+/Le+	−1.049	<0.001
DFLNHa	Se+/Le+	−0.777	<0.001
LNDFH-I	Se+/Le+	−0.212	0.065
LNFP-I	Se+/Le+	−0.408	<0.001
LNFP-III	Se+/Le+	−0.203	<0.001
LNH	Se+/Le−	−0.571	0.003
LNH	Se+/Le+	−0.580	<0.001
Hex4HexNAc2	Se+/Le+	−0.693	<0.001
LNnDFH	Se+/Le+	0.370	0.036
LNnFP-V	Se−/Le+	−0.480	0.004
LNnFP-V	Se+/Le+	−0.470	<0.001
LNnT	Se+/Le+	−0.604	<0.001
LNT	Se+/Le+	−0.283	0.025
LSTc	Se−/Le+	−1.269	<0.001
LSTc	Se+/Le−	−1.352	0.059
LSTc	Se+/Le+	−1.263	<0.001
MFLNH-III	Se+/Le+	−0.836	<0.001

Only significant and adjusted-for-multiple-testing changes are reported in the table. 2'FL, 2'-O-Fucosyllactose; 3FL, 3-O-Fucosyllactose; 6'SL, 6'-O-Sialyllactose; DFLNHa, Difucosyllacto-N-hexaose a; LNDFH-I, Lacto-N-difucohexaose-I; LNFP-I,–III, Lacto-N-fucopentaose-I,-III; LNH, Lacto-N-hexaose; Hex, Hexose; HexNAc, N-acetylhexosamine; LNnDFH, Lacto-N-neodifucosylhexaose; LNnFP-V, Lacto-N-neofucopentaose-V; LNnT, Lacto-N-neotetraose; LNT, Lacto-N-tetraose; LSTc, Sialyllacto-N-tetraose c; MFLNH-III, Monofucosyllacto-N-hexaose-III,, Human Milk Oligosaccharide; q-value, level of confidence after correction for multiple HMO testing.

of which are known to contain the structural features α-1,3-fucose or α-1,4-fucose which are dependent on the activity of FUT3. The HMOs in cluster 5 are all present at significantly higher concentrations in milk group 2 and milk group 4 (**Supplementary Table 1**, $p < 0.05$).

Representation of the Milk Groups Among Individuals

In order to better understand the relationship between HMOs in lactating mothers at 3 months of lactation, exploratory principal component analysis (PCA) was performed and was found to explain more than 80% of the variance. The first component explained 62.2% of the individual variability and is mainly driven by 3FL and LNFP-II in one direction and by 2′FL and LNFP-I in the other. The first component separates the whole population in 3 distinct groups, which are Se+/Le+, Se+/Le−, and Se−/Le+ (**Figure 3**). The second component explained 19.5% of the individual variability and is mainly driven by A-tetrasaccharide (A-tetra). Both the Se+/Le+ and the Se+/Le- groups can be separated in the second dimension, while the Se−/Le+ group is unaffected (**Figure 3**). This makes perfect sense with regards to the A-tetra HMO structure [GalNAcα1-3(Fucα1-2)Galβ1-4Glc], as A-tetra can only be produced by individuals who are Se+ and are blood group A (32).

Genotypes and Secretor/Lewis Status in the Population Under Study

FUT2 and *FUT3* exons were sequenced to identify functional single nucleotide polymorphisms (SNPs) affecting HMO profiles in the milk of 156 mothers. Initially, 2,458 SNPs were identified and, after quality check, 20 coding variants were identified in the population (**Table 2**). The presence of a homozygous genotype for one of the functional variants (i.e., mutation has an effect on enzymatic activity) known to affect Secretor and Lewis status was used to assign mothers as Secretor or non-Secretor and as Lewis positive or Lewis negative. No homozygous mothers were identified for the minor alleles of rs1047781 or rs200157007 in the cohort samples. Therefore, the Secretor status was defined based on SNP rs601338 only. Overall, 134 (88.3%) samples were identified as Secretors and 18 (11.8%) as Non-Secretors.

Lewis status is known to depend on SNPs, rs3745635, rs28362459, rs3894326, and rs812936. In our population, no rs3745635 variants were identified. Therefore, the Lewis status was defined based on SNPs rs28362459, rs3894326, and rs812936. In our study, 9 (5.9%) samples were identified as Lewis negative, and 143 (94.1%) samples were identified as Lewis positive. In 7 samples, rs812936 was found in its minor allele monozygous form C/C and in 2 other samples, rs28362459 was in its minor allele homozygous form G/G. No minor alleles homozygous for rs3894326 were found in the population.

Haplotype analysis for *FUT2* and *FUT3* regions revealed a high linkage disequilibrium LD, $r^2 > 0.8$ (**Supplementary Figure 1**).

HMO Concentrations Are Highly Associated With Genetic Variants

At 3, 6 and 12 months post-partum, we found 2′FL concentrations below LoQ in 13.4, 15.9, and 10.7% of the mothers, respectively. LNFP-II concentrations at 3, 6, and 12 months were below LoQ in 10.8, 12.6, and 14.7% of the mothers, respectively. Secretor and Lewis status based on HMO concentrations did not differ among the 3 time-points for the same individual. We sought to correlate HMO concentrations with *FUT2* and *FUT3* non-functional variants. Concentrations of individual HMOs dependent on Secretor or Lewis status were associated with *FUT2* and *FUT3* genotypes of the individual, respectively. Rs601338 was significantly associated with the concentrations of both 2′FL and LNFP-I in breast milk (**Figure 4**). Individuals with the wild type G/G genotype have high concentrations of 2′FL in their milk (**Figure 4**). Indeed, for most samples identified as Non-Secretors by the presence of rs601338 variations, 2′FL concentrations were below LoQ <20 mg/L. Two samples genetically identified as Secretors (G/G) also had 2′FL concentrations below LoQ. A single sample was indentifed as Non-Secretor (A/A) and 2′FL concentration was higher than LoQ. For both results, there was no match between the genetic and the HMO analysis. We lowered the threshold of MAF to 1% to identify rare and potentially missense variants to

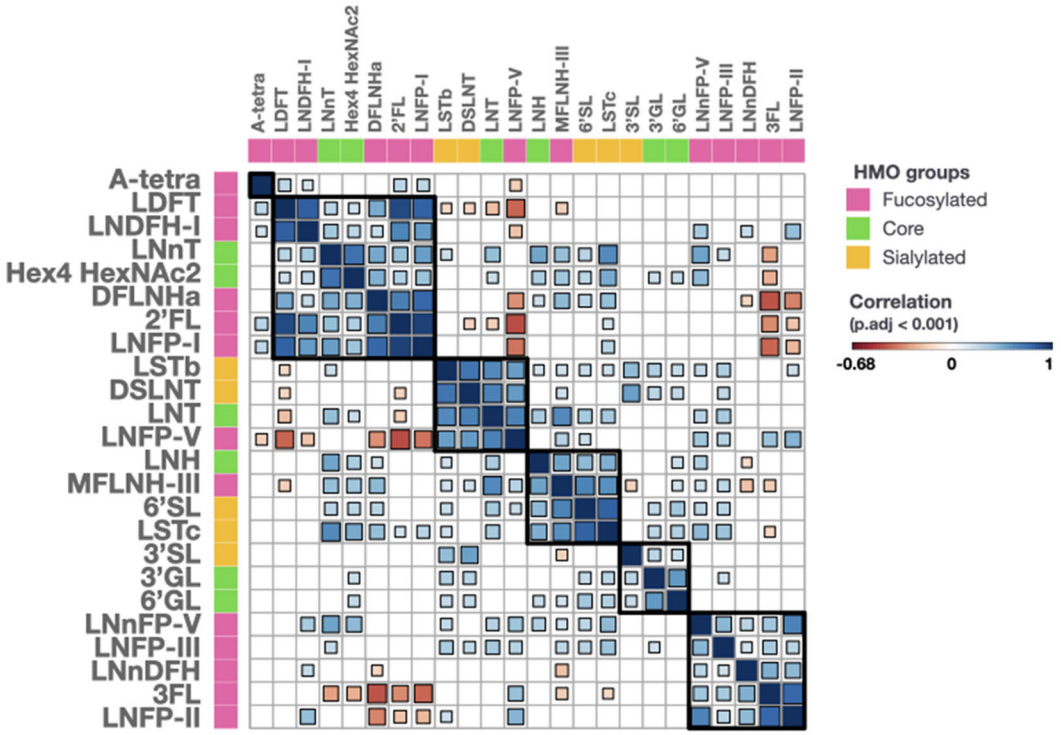

FIGURE 2 | Correlations of all measured HMOs. HMOs are classified into 3 groups, fucosylated (pink), core (green) and sialylated (orange). Correlation values range from −0.68 (dark red) to 1 (dark blue). Size and color of a square are proportional to the correlation coefficient value. Squares are only shown when the adjusted for multiple testing p-value is below 0.001. 2'FL, 2'-O-Fucosyllactose; 3FL, 3-O-Fucosyllactose; 3'GL, 3'-O-Galactosyllactose; 3'SL, 3'-O-Sialyllactose; 6'GL, 6'-O-Galactosyllactose; 6'SL, 6'-O-Sialyllactose; A-tetra, A-tetrasaccharide; DFLNHa, Difucosyllacto-N-hexaose a; DSLNT, Disialyl-lacto-N-tetraose; Hex, Hexose; HexNAc, N-acetylhexosamine; LDFT, Lactodifucotetraose; LNDFH-I, Lacto-N-difucohexaose-I; LNFP-I,-II,-III,-V, Lacto-N-fucopentaose-I,-II,-III,-V; LNH, Lacto-N-hexaose; LNnDFH, Lacto-N-neodifucosylhexaose; LNnFP, Lacto-N-neofucopentaose; LNnFP-V, Lacto-N-neofucopentaose-V; LNnT, Lacto-N-neotetraose; LNT, Lacto-N-tetraose; LSTc, Sialyllacto-N-tetraose c; MFLNH-III, Monofucosyllacto-N-hexaose-III.

explain these results, but we did not identify any novel FUT2 SNPs in this population located at exon regions (data not shown). Similar results as for 2′FL were observed for LNFP-I, another major FUT2-dependent HMO (**Figure 4**).

Rs812936, the main genetic determinant of Lewis status, was associated with LNFPII concentration. Indviduals with G/G genotype had higher concentrations of LNFPII in their milk (**Figure 5**). For the 8 samples identified as Lewis negative based on rs28362459 and rs812936 SNPs, LNFP-II was not detected in the milk. However, in the population, 8 samples were identified as Lewis positive based on rs28362459, rs3894326, rs812936 (wild type allele homozygotes or heterozygotes), but had no detectable LNFP-II in the respective milk samples. For 6 of them, two or more missense *FUT3* variants were in their heterozygote form with rs812936 and rs778986 always in their heterozygote form T/C and C/T, respectively. From our data, we could not provide an explanation for the remaining 2 samples.

Genetic Predictors of Milk Groups

There were genetic polymorphisms in the *FUT2* and *FUT3* genes that were associated with the concentrations of major HMOs. We showed that rs516246, rs516316, rs492602, rs681343, and rs601338 were associated with low concentrations of 2′FL, and LNFP-I while being associated with high concentrations of 3FL and LNFP-II. On the other hand and to a lesser extent, rs28362459, rs3894326, rs3760776, rs812936, rs778986, rs1800022, rs3745635, and rs1800027 were associated with high concentrations of 2′FL and LNFP-I and low concentrations of 3FL and LNFP-II. Interestingly, rs128362465 had a tendency to be associated with A-tetra concentrations (**Figure 6A**).

In order to predict the milk group of future mothers, we fitted a MLP model based on genetic polymorphisms and showed that rs812936, rs778986, rs681343, rs601338, rs28362459 were top-predictors of the milk groups with an average accuracy of 0.978 (**Figure 6B**, **Supplementary Table 3**).

A Genetic Score to Predict 2′FL Concentrations in Milk for Future Secretor Mothers

We developed a genetic score to demonstrate the additive impact of genetic polymorphisms of *FUT2* and *FUT3* genes on the concentrations of 2′FL, the most abundant HMO in Secretor milk. Interestingly, we showed that the Secretor population could be divided into two sub-populations with moderate and high levels of 2′FL, and that we could predict fairly well the

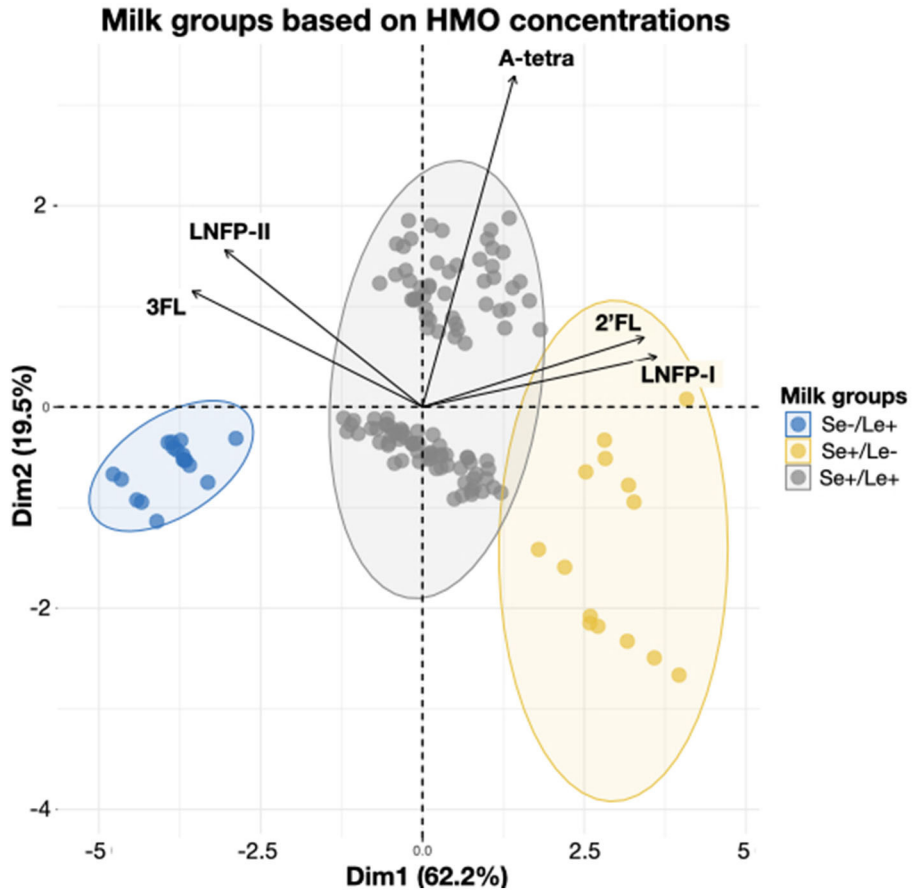

FIGURE 3 | Individual mothers grouped based on 5 HMO concentrations at 3 months. The HMOs concentrations are used to determine the milk group as defined in (22). Secretor-positive groups are even further separated by the presence or absence of A-tetra. Ellipses represent the 95% percentile of confidence.

2′FL concentrations in a mother's milk based on her genetic score (Adjusted-$R^2 = 0.58$, $p < 6.6.10^{-9}$). A zero or negative score predicted a moderate 2′FL concentration, while a positive score predicted a high amount of 2′FL in her milk (**Table 3** and **Figures 7A,B**). Though 2′FL concentrations were mainly associated with rs601338 polymorphism and heterozygous mothers were predominatly represented in the moderate level group, our polygenic score significantly outperformed predictions based on rs601338 alone ($p < 0.001$).

DISCUSSION

Our study provides for the first time detailed and systematic insight on the link between the breast milk HMO concentrations and maternal *FUT2* and *FUT3* genetic variants. We focused our analysis on exonic genetic variants to identify the ones with a functional role on FUT2 and FUT3 enzyme activities. In this population, we identified 1 known missense SNP on *FUT2*, rs601338, responsible for the non-Secretor phenotype in milk and 3 known missense SNPs, rs28362459, rs3894326, and rs812936 on *FUT3*, responsible for the Lewis negative phenotype in milk.

SNP rs601338, the predominant *FUT2* variant has a minor allele frequency (MAF) in European and African populations ranging from 30 to 57% (1,000 Genomes Project, https://www.internationalgenome.org/1000-genomes-browsers). This SNP has a very low frequency 0–1% in South East Asian populations, whereas in Indian populations, the average MAF is 25% (1,000 Genomes Project). Generally, in our population the samples genetically identified as non-Secretors had an A/A genotype and, as expected, measured 2′FL and LNFP-I concentrations below LoQ. Two samples genetically identified as Secretors G/G also had 2′FL and LNFP I below LoQ, actually close to their LoD (limit of detection) of 3.9 and 2 mg/L for 2′FL and LNFP-I, respectively (29). We investigated our dataset by looking for novel rare *FUT2* non-coding variants, but we did not identify any that could explain these results. It is possible that other *FUT2* SNPs outside the exonic regions, perhaps with a regulatory function could define the enzyme expression. To our knowledge, the FUT2 enzyme is the only known fucosyltransferase that generates α-1,2-fucosylated HMOs. Hypothetically, another unknown fucosyltransferase may be active in the mammary gland explaining our observation of 2 samples identified genetically as Secretors, but expressing only very small amount of 2′FL.

TABLE 2 | The single nucleotide polymorphisms (SNPs) identified in the population after FUT2 and FUT3 exome sequencing.

SNP	CHR	chrpos	MAF	Function	Allele	AA change	Position in protein	Affect secretor/Lewis status
rs3894326	FUT3	5843773	0.07895	Mis.	A>C/T	I>K	356	Yes
rs28362465	FUT3	5844228	0.01316	Syn.	T>C/G	S>S	NA	No
rs3745635	FUT3	5844332	0.02961	Mis.	G/A	G>S	170	Yes
rs778986	FUT3	5844526	0.1908	Mis.	A>G	T>M	105	Yes
rs812936	FUT3	5844638	0.2007	Mis.	G>A/C	W>R/G	68	Yes
rs28362459	FUT3	5844781	0.125	Mis.	A>C/G/T	L>R	20	Yes
rs145362171	FUT3	5844793	0.01645	Mis.	C>G	C>S	16	NA
rs516316	FUT2	48702888	0.3553	IV	G>C	NA	NA	NA
rs516246	FUT2	48702915	0.3553	IV	C>T	NA	NA	NA
rs492602	FUT2	48703160	0.352	Syn.	A>G	A>A	68	No
rs681343	FUT2	48703205	0.3553	SG	C>A/T	Tyr	83	Yes
rs281377	FUT2	48703346	0.4507	Syn.	C>T	N>N	130	No
rs1800022	FUT2	48703368	0.01645	Mis.	C>T	R>C	138	No
rs601338	FUT2	48703417	0.3553	SG	G>A	W>*Ter	154	Yes
rs1800027	FUT2	48703469	0.102	Mis.	C>G/T	H>Q	171	Yes
rs602662	FUT2	48703728	0.4046	Mis.	G>A	G>S	258	Yes
rs141630650	FUT2	48703844	0.02303	Syn.	A>A	A>A	296	No
rs485186	FUT2	48703949	0.4046	Syn.	A>G	T>T	331	Yes
rs485073	FUT2	48703998	0.4046	3UV	A>G	NA	NA	NA
rs603985	FUT2	48704000	0.4046	3UV	T>C	NA	NA	NA

chr, chromosome; chrpos, chromosome position; MAF, Minor Allele Frequency; AA, amino acid; Mis, missense; Syn, synonymous; NA, not analyzed; IV, intron variant; SG, stop gain variant; Ter, termination; FUT2/3, Fucosyltransferase 2/3; 3UV, 3' Untranslated region.

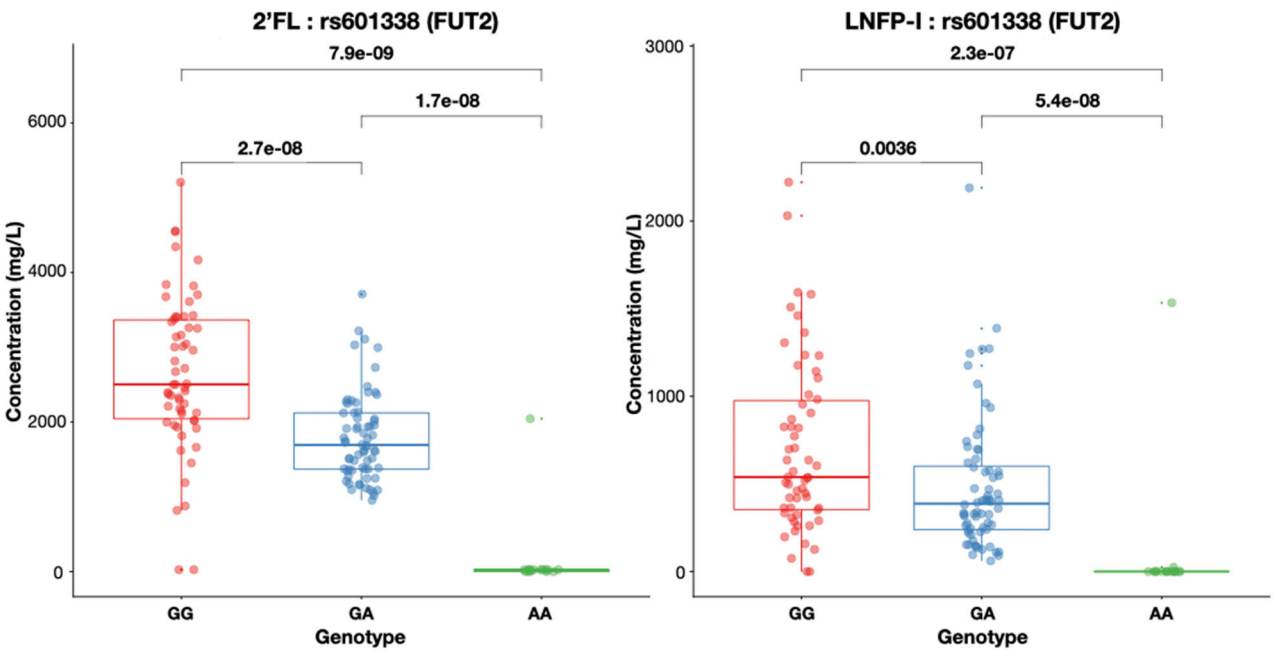

FIGURE 4 | Both 2'FL and LNFP-I concentrations in mg/L in breast milk are significantly different (Mann–Whitney test for non-matched non-parametric data: $P < 0.0001$) depending on rs601338 genotypes. Box plots with median and 25 and 75% interquartiles and 95% CI are shown.

Another possibly more likely explanation is the presence of mutations in regulatory elements for *FUT2* that we did not capture in our analysis. A future approach would be to perform an expression quantitative trait loci (eQTL) to identify *cis*- or *trans*-eQTLs that may affect the expression of the *FUT2* gene and then verify this by testing their correlation with

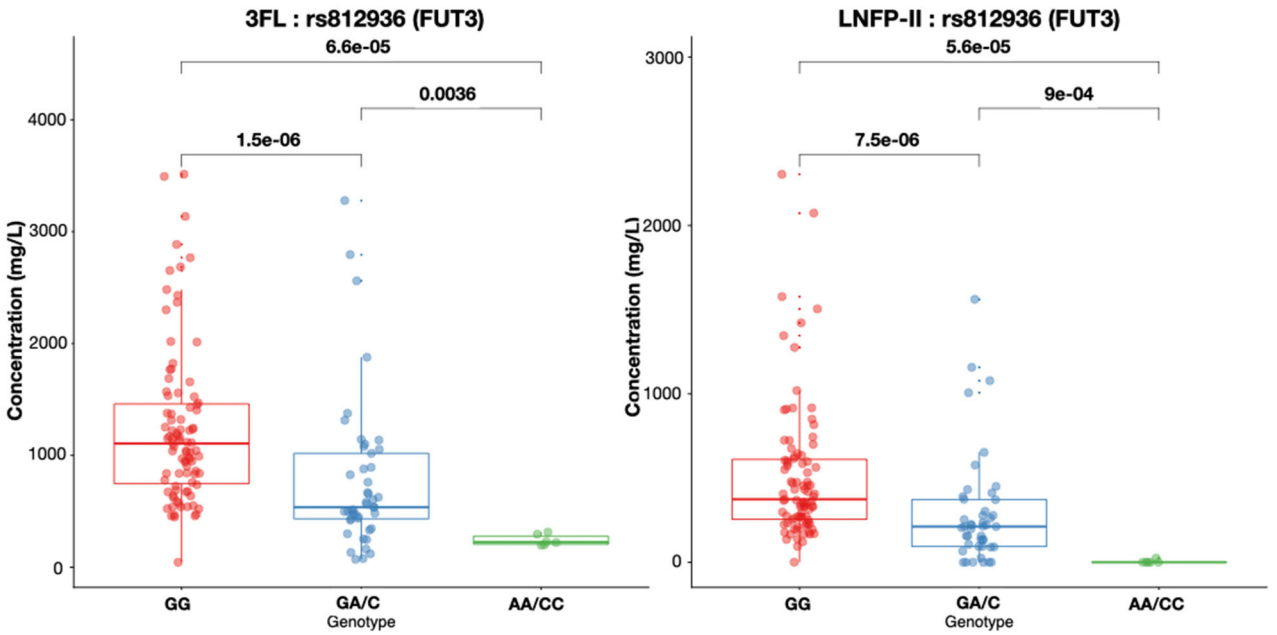

FIGURE 5 | 3FL and LNFP-II concentrations in mg/L in breast milk according to rs812936 genotypes. Box plots with median and 25 and 75% interquartiles and 95% CI are shown.

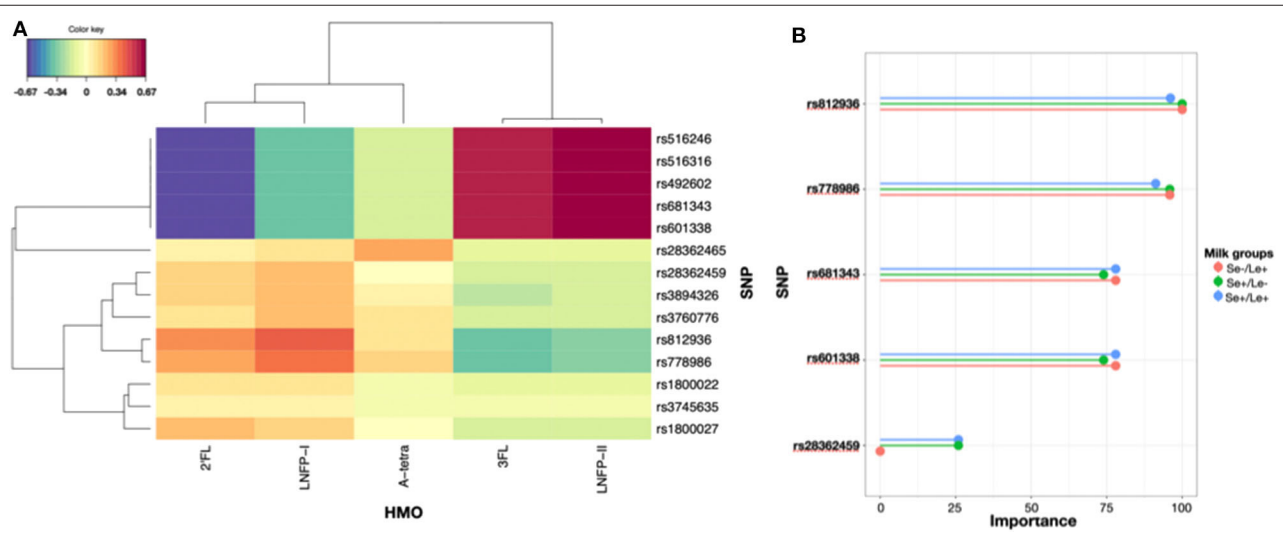

FIGURE 6 | (A) sPLS-selected genetic polymorphisms and their associations with HMO levels expressed as a distance between genotypes and HMO levels in 142 individuals. **(B)** Importance of genetic polymorphisms in predicting the milk group of mothers with a MultiLayer Perceptron model.

2′FL concentrations. Furthermore, a genome wide approach to find genetic variants associated with 2′FL concentrations could provide further insight into the unknown variation controlling the expression of the enzyme.

We used the same approach to identify the link between *FUT3* exonic variants and α1,3-4-fucosylated HMO, like LNFP-II and 3FL. In our population, most of the Lewis negative mothers were homozygotes for one of the three functional *FUT3* SNPs rs28362459, rs3894326, rs812936. In contrast to Secretor status, the Lewis status seems to be defined by a higher number of SNPs which are also less well characterized than *FUT2* (33, 34). SNP rs28362459 occurs more frequently in South East Asian, Indian and African populations (MAF = 25 to 35%) compared to European populations (MAF = 10%). SNP rs3894326 is more common in Asian populations (MAF = 15%) compared to European and African (MAF > 7%). Finally, SNP rs812936 is not frequent in South East Asian populations (MAF = 3%) but ranges from 10 to 20% in all

other populations included in the 1000 Genomes Project 1000 Genomes Project, (https://www.internationalgenome.org/1000-genomes-browsers). In our cohort, we identified 8 mothers as Lewis positive based on the three SNPs rs28362459, rs3894326 and rs812936, yet no LNFP-II was detected in their milk. Interestingly, 6 of them were heterozygotes for both rs812936 and rs778986 SNPs, indicating that the phenotype may be a result of compound heterozygosity (35). However, we could not analyze phased genotypes in our dataset to explore this possibilty, due to high linkage disequilibrium in the region. Future studies need to explore whether *FUT2* and *FUT3* genetic variants are subjected to compound heterozygosity explaining the missing production of specific HMOs despite an apparent functional genotype.

In this population of European mothers, those with the heterozygous forms of the Secretor and Lewis status-defining SNPs appear to have intermediate levels of the dependent HMOs like 2′FL, LNFP-I, LNFP-II, and 3FL compared to higher levels in the major allele homozygous functional forms. This may have several implications for research on HMO biology and clinical relevance and may explain part of the large variability observed for many HMOs in breast milk. In an attempt to further explain variability of HMOs by combined *FUT2* and *FUT3* variations, we constructed a genetic score to predict the concentrations of 2′FL. Indeed, we observed that 5 SNPs located in both genes were able to predict 2′FL concentrations in breast milk. The finding did not seem to be solely dependent on presence of *FUT2* non-functional SNP. Instead, the combination of both *FUT2* and *FUT3* variation were needed to explain the variability in 2′FL levels, confirming the hypothesis that final concentrations of specific individual HMOs are influenced by the balance between FUT2 and FUT3 expression (1), as well as donor and acceptor substrate availability for the respective enzymes.

Similarly, our results showed that 5 *FUT2* and *FUT3* SNPs are sufficient to predict the milk groups in the population. Our population was homogenous with European ancestry and it would be important that these relationships between *FUT2* and *FUT3* genetic variants and HMOs are confirmed in admixed or populations with different ancestry, e.g., Asian (17).

We also report here how HMOs cluster and change in concentration over the course of lactation until 12 months of age. Albeit at 12 months of age our sample size was relatively small ($N = 28$), these data still provide a valuable complement to previously published studies reporting concentrations of HMOs beyond 6 months of lactation that generally had even lower sample sizes (36, 37). Overall concentrations for most HMOs decrease over time of lactation with some changes

TABLE 3 | Summary table for the SNPs included in the genetic score.

SNP	Coefficient	Standard error	*P*-value
rs601338	−0.56304	0.06936	<0.001
rs28362459	0.39504	0.06534	<0.001
rs778986	0.18314	0.04536	<0.001
rs1800022	−0.36211	0.14316	0.013
rs281377	0.10313	0.05702	0.074

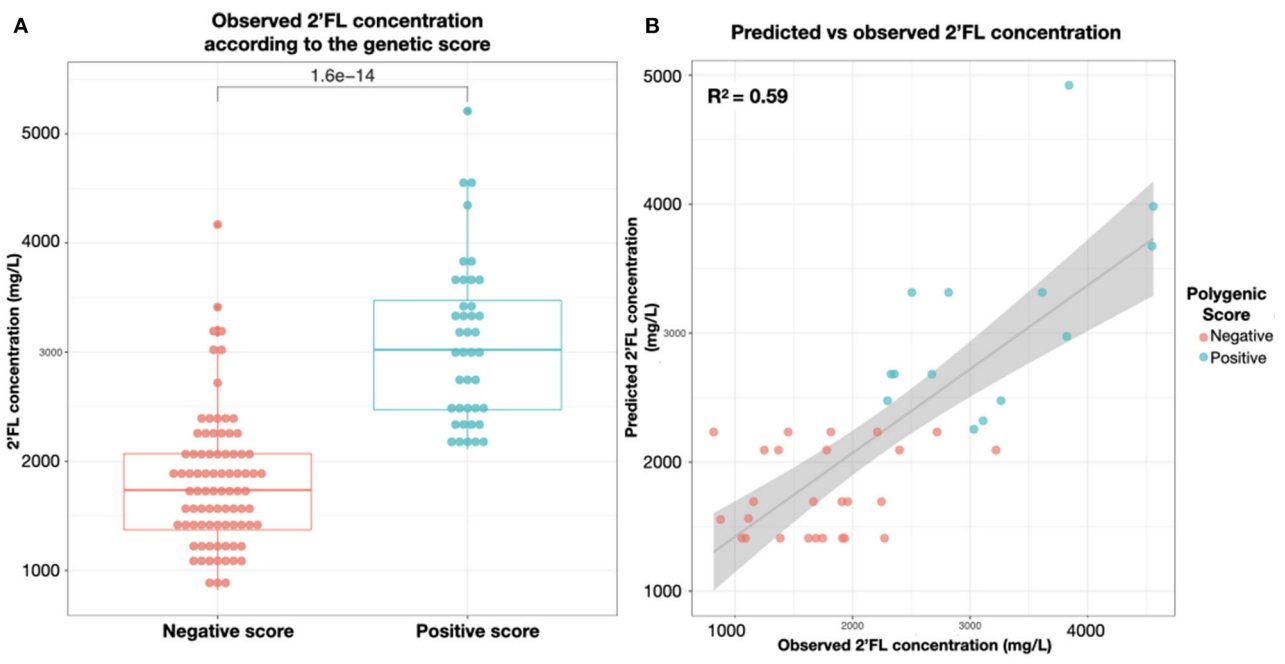

FIGURE 7 | (A) 2′FL concentrations in milk (mg/L) of the 124 secretor mothers by genetic score categorization in high secretors positive score and moderate secretors zero or negative score. **(B)** In an independent test dataset, correlation between genetic score predicted and observed concentrations of 2′FL (mg/L) secreted in the milk of the individual mothers.

being statistically significant like for 6′SL, LST-c, and MFLNH-III. On the other hand, concentrations of 3FL and LNnDFH increase from 6 to 12 months. Certain HMOs are highly correlated with each other like the FUT2-dependent-HMOs, which are inversely correlated with FUT3-dependent and some sialylated HMOs. Overall the results reflect the dependence on specific fucosyltransferases and the substrate competition for these enzymes reported before (1). We observed several clusters showing some expected relations between HMOs, like a FUT2 or FUT3 dependence, but the clusters also showed some unexpected relations. For example, LNnT clustered with the FUT2 dependent HMOs 2′FL and LNFP-I and 3′SL clustered with the galactosyllactoses 3′GL and 6′GL. Very few studies have analyzed HMOs up to 12 months of lactation (37). Gridneva et al. (37) reported that although total measured HMOs slightly decreased over time, this is not statistically significant, a result similar to ours. Individual HMOs or groups of HMOs, however, may have a more dynamic profile over time, but this was not reported in that study. Generally, for HMOs like 3FL, the increase in concentration over time may reflect a role relevant to later developmental stages. Yet, today no such associations were reported in the literature as far as we know.

We found that genetic characterization by milk groups was a strong factor explaining the HMO distribution and that 5 individual HMOs 2′FL, LNFP-I, A-tetra, 3FL, and LNFP-II were sufficient to characterize these clusters. Within the clusters, smaller subgroups were visible, mainly driven by A-tetra. This may explain a recent report showing that within Secretors smaller subgroups are present (38). Actually, A-tetra appears only in milk of Secretor mothers, who are also of the blood group A type meaning they have a functional N-acetylgalactosamine transferase that can add GalNAc to H-type glycans like 2′FL for example (32).

Clearly, HMO concentrations are strongly determined by genetic factors, namely SNPs on *FUT2* and *FUT3* and their combinations. Consequently, these factors should be considered when exploring HMO compositional variation in relation to other maternal factors and diet. Yet, unidentified rare variation and organization of genomic regions need also to be further explored and possibly accounted for. In addition, further large studies are needed to identify currently unknown regulatory variations that may impact the function of these fucosyltransferases or other enzymes involved in the production of HMOs. Such additional factors may be able to better explain the temporal dynamic changes and the large inter-individual variability seen in several observational studies. Ultimately, information explaining HMO variability is important to better understand and interpret HMO effects observed in relation to growth and health measures in breastfed infants at different developmental stages, as some like the maternal genetic factors are also linked to the infants genetic makeup.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA643141.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Leipzig Faculty of Medicine. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

GL conducted statistical analysis and drafted the manuscript. MS conducted statistical analysis and reviewed the manuscript. AC conducted laboratory analysis and reviewed the manuscript. JM designed and conducted laboratory analysis and reviewed the manuscript. MV and WK designed the study and reviewed the manuscript. TK analyzed data and reviewed the manuscript. SA designed the study and drafted the manuscript. NS conceived and designed the analysis and drafted the manuscript. AB concieved and designed the study and drafted the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2020.574459/full#supplementary-material

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

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ORIGINAL RESEARCH
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Circulating Branched Chain Amino Acid Concentrations Are Higher in Dairy-Avoiding Females Following an Equal Volume of Sheep Milk Relative to Cow Milk: A Randomized Controlled Trial

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Background: Intolerances to bovine dairy are a motivating factor in consumers seeking alternate—or replacement—dairy beverages and foods. Sheep milk (SM) is an alternate dairy source, with greater protein, although similar amino acid composition compared to cow milk (CM). Studies are yet to address the appearance of circulating amino acids following consumption of SM, relative to CM, in humans.

Objective: To clinically determine the appearance of branched chain amino acids, and other amino acids, in circulation in response to equal servings of SM and CM, in females who avoid dairy products.

Design: In a double-blinded, randomized, cross-over trial, 30 self-described dairy avoiding females (20–40 years) drank 650 mL of SM or CM that were reconstituted from the spray dried powders (30 and 25 g in 180 mL water, respectively) on separate occasions, following an overnight fast. After reconstitution, the energy and protein provided by SM was higher than for CM (2,140 vs. 1,649 kJ; 29.9 vs. 19.4 g protein); content of branched chain amino acids (BCAAs) were 10.5 and 6.5 mg·mL^{-1}, respectively. Blood samples were collected at fasting and at regular intervals over 5 h after milk consumption. Plasma amino acids were measured by HPLC.

Results: 80% of subjects self-identified as lactose intolerant, and the majority (47%) "avoided drinking milk" "most of the time". SM resulted in greater plasma appearance of BCAAs at 60 min (641.1 ± 16.3 vs. 563.5 ± 14.4 μmol·L^{-1}; $p < 0.001$) compared with CM. SM similarly resulted in elevated postprandial concentrations of the amino acids lysine, methionine, and proline, particularly at 240 min (time × milk interactions $p = 0.011$, 0.017, and $p = 0.002$, respectively). Postprandial increases in plasma alanine concentrations were sustained to 120 min after CM (time × milk interaction $p = 0.001$) but not after SM, despite greater quantities provided by SM.

Conclusions: SM is a rich source of protein, and relative to CM, provides a greater quantity of BCAAs, with a corresponding elevation of the postprandial circulating BCAA response. SM is therefore a possible dairy alternative of benefit to those who need to increase total protein intake or for individuals with heightened protein requirements.

Unique Identifier and Registry: https://www.anzctr.org.au/Trial/Registration/TrialReview.aspx?id=375324, identifier U1111-1209-7768

Keywords: ovine milk, bovine milk, protein digestion, milk alternative, essential amino acids, postprandial, adult nutrition

INTRODUCTION

Avoidance of dairy products is prevalent among some populations. In Australia, dairy avoidance may be practiced by 17% of the population, of whom the majority (13%) avoid dairy on the basis of self-reported negative reactions to dairy products (1). This trend is evident globally (2, 3), where perceived or actual intolerance to cow milk (CM)—either due to lactose or other components of milk (4)—influences consumption behaviors (5, 6). Dairy products are recognized as an important source of nutrients (7, 8), including calcium and high quality protein (7), essential for growth and development. Nutritionally, dairy avoidance in the context of a Western diet has the potential to increase the risk of nutrient insufficiencies, contributing to poor health in the form of low bone mineral density and even chronic illness (8, 9). In populations for whom dairy products can be a rich source of energy and protein, such as young children (10) or the elderly (11), it is important to identify suitable dietary alternatives to bovine dairy. While non-dairy alternatives exist, these are often not nutritionally equivalent to dairy, increasing the risk of dietary deficits of key dairy-derived nutrients for dairy avoiders (9). For instance, certain plant-based milks are lacking in essential amino acids (12), with plant proteins contributing less protein accretion than milk proteins (13).

Sheep (ovine) milk (SM), while compositionally different to CM, is an alternate ruminant milk which maintains many of the key nutritional features of bovine milk. Anecdotal evidence that milk from other ruminants (goat and sheep) is easier to digest than bovine milk (14), particularly for those with intolerance to dairy products (15), has been cited to support the use of SM as a more suitable alternative to CM. Compared to cow milk, sheep milk has a higher total solids content, accounted for by greater protein (6.2 vs. 3.2 %) and fat (7.9 vs. 3.6 %) content (16). On a weight (mg/g protein) basis, sheep milk has similar essential amino acid (AA) content as bovine milk (17). However, there are sequence differences of key proteins, including α_{s1}-, α_{s2}-, β-, and κ-caseins (18) and whey proteins (19). This leads to the structural and physiochemical properties of SM when milk is acidified (e.g., in processing or in human stomach) (16). These types of physicochemical effects have been shown to influence digestion kinetics for bovine milks (20), a factor which impacts on AA appearance in circulation (21, 22) and postprandial protein accretion (22, 23). Although in-depth descriptions of SM digestion kinetics are limited (24, 25), a rodent model demonstrated higher ileal amino acid digestibility in rats fed SM relative to CM, resulting in greater circulating concentrations of leucine, lysine, and methionine (26). Thus, SM with greater protein composition and greater ileal digestibility than CM may offer relative advantages in meeting protein needs. This may be of particular importance for those with low protein intake or who have greater protein requirements, including those who habitually avoid bovine dairy. Studies are yet to address the differences in protein digestion and AA appearance in human subjects.

The aim of this study was to compare, on a "portion-for-portion" basis, the appearance in circulating BCAA (i.e., leucine, valine and isoleucine) following ingestion of sheep or cow milk, and further to compare the appearance of other AA in circulation. CM composition is consistent throughout the year due to continual production and large volumes. However, SM is currently a niche product, with a seasonal production pattern in New Zealand. The composition of SM varies throughout the season, notably with an increase in fat and protein content toward the end of the season (16, 27). In addition, there is a difference in milk composition between producers (27), across sheep breeds (28, 29) and in geographical regions (17, 28, 29). Thus, spray dried powdered milk was selected as a shelf stable milk preservation technique that was suitable for both sheep and cow milk. To improve the generalisability of the results, reconstituted sheep milk powder from two different producers and commercially available reconstituted cow milk powder were used as the test drinks in this study.

We hypothesized that circulating BCAAs and other AA concentrations would be greater following the ingestion of SM compared to CM.

METHODS

Experimental Design

The study was a double-blinded, randomized and crossover study, with equal allocation ratio and an equivalence framework. The study was conducted at the Clinical Research Unit, Liggins Institute, University of Auckland between July and November 2018. Postprandial appearance of BCAA at 60 min (peak appearance) was compared between sheep and cow milk as the primary outcome. Secondary outcomes of the postprandial appearance over 4 h of all amino acids were also compared. Additional secondary outcomes of lipid responses, lactose

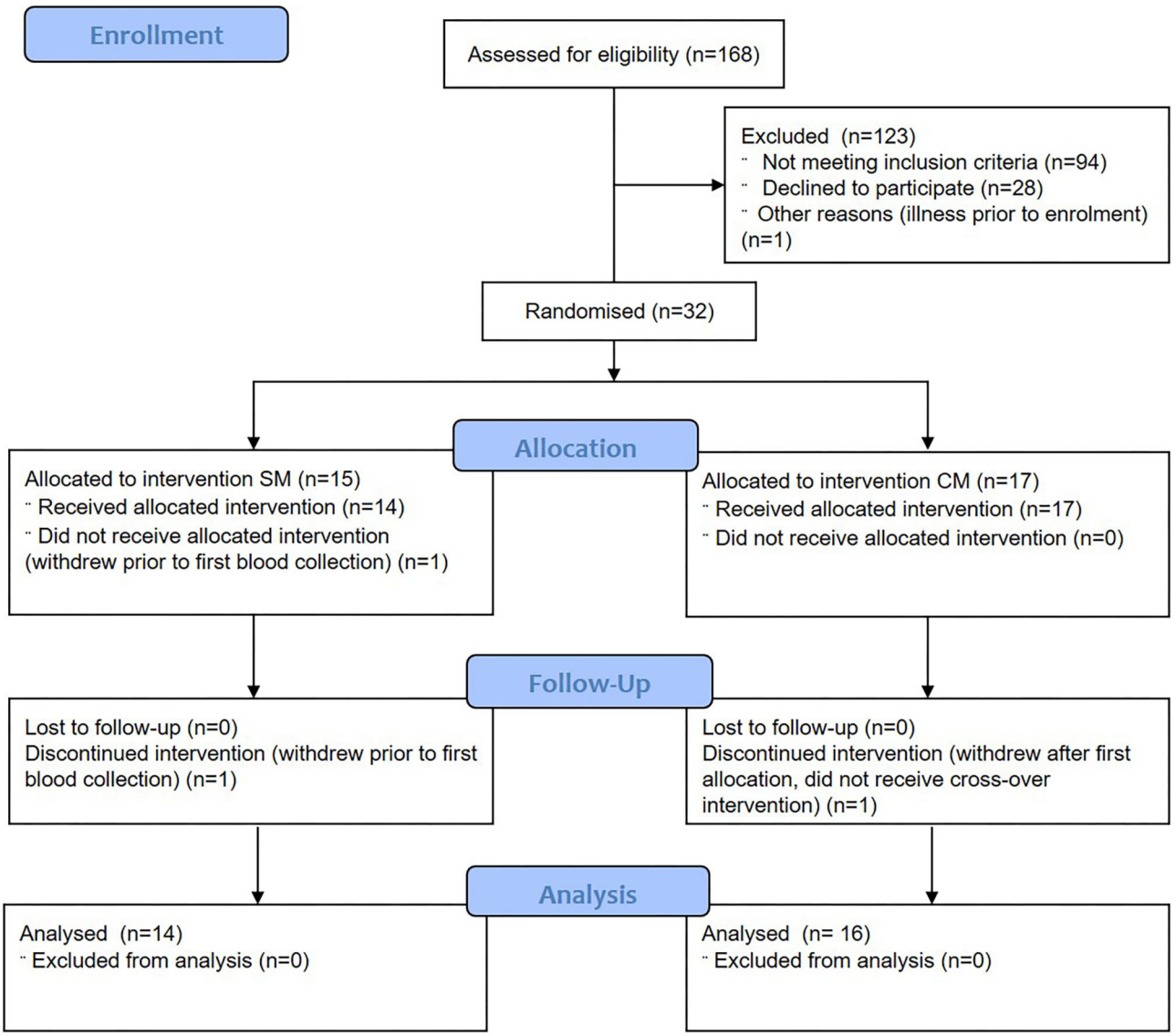

FIGURE 1 | Consolidated Standards of Reporting Trials flow diagram of participant eligibility, enrolment, randomization, follow-up, and analysis. SM, sheep milk; CM, cow milk.

malabsorption, subjective appetite and digestive comfort were also collected, but have not been reported here.

The study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the New Zealand Health and Disability Ethics Committees (Reference no. 18/NTB/92). The trial was prospectively registered with the Australian New Zealand Clinical Trials Registry (ACTRN12618001030268). Written informed consent obtained from eligible participants prior to study commencement.

Participants

Females ($n = 32$) aged 20–40 years were recruited using print and electronic advertisements. Two subjects withdrew prior to the completion of the protocol (**Figure 1**). Participants were required to self-describe to "avoid drinking milk" as a binary classification (yes/no) and have a BMI between 18 and 28 kg/m^2. Participants were not eligible if they had a known allergy to milk, or known gastrointestinal (celiac, or inflammatory bowel disease) or metabolic disease, or were currently taking medications expected to interfere with normal digestive and metabolic processes including proton pump inhibitors, laxatives, antibiotics or prebiotics (within last 3 months). Participants with pre-existing cardiovascular diseases or self-reported alcohol intake >28 units per week were also not included.

Study Procedures

Following determination of eligibility, subjects were randomized to receive either sheep milk or cow milk in a cross-over manner at least one week apart. Randomization sequences were

computer-generated by www.randomizer.org (30). Allocation was implemented through sealed envelopes. Investigators and participants were blinded to treatment identity and until primary outcome data analysis was complete.

Subjects were asked to attend the Clinical Research Unit on two occasions, separated by at least one week. Prior to the visits, subjects completed a validated lactose intolerance screening questionnaire (31), along with questions about habitual dairy consumption patterns. On the day preceding each visit, subjects were asked to avoid foods high in fat, alcohol, caffeine and taking part in any strenuous exercise. Subjects were provided with a standardized low-fiber, low-fat meal on the day preceding the visit to consume as their evening meal.

Subjects arrived the following morning, after an overnight fast, excluding water, from 10 pm the night prior. A venous cannula was inserted, and a fasting blood sample collected. Subjects then consumed 650 mL of the assigned milk, within 10 min.

Following milk ingestion, blood samples were collected at 15 min intervals for the first 90 min, and then hourly starting at 2 h for 4 h post-ingestion. During the 4 h, subjects refrained from the consumption of any additional food or drink except water.

Blood pressure was assessed at fasting at each acute visit by automatic blood pressure monitor (Heart Sure BP100, Omron, Kyoto, Japan). Height (by stadiometer to the nearest 0.1 cm; Holtain Ltd., Crymych, Dyfed, UK), weight (by digital weighting scale to the nearest 0.1 kg; Tanita® 1582 Medical Scale, Wedderburn, Auckland, New Zealand) and waist circumference (by non-flexible tape measure) were measured on the first visit only by standard procedures in the fasted state after voiding.

Blood Collection

Venous blood samples were collected in EDTA containing vacutainers (Becton Dickinson & Company, Mount Wellington, New Zealand), and plasma was removed after centrifugation at 2,000 x *g* for 15 min at 4°C and frozen at −80°C prior to analyses.

Questionnaires

Demographic information about dairy consumption behaviors (avoidance, restriction, frequency of symptoms experienced) was collected using categorical scales. The frequency of milk consumption was assessed as serves per day, and habitual frequency by food frequency questionnaire (FFQ) category scale from the EPIC-Norfolk FFQ (32). Perceived lactose intolerance was assessed using a validated visual analog scale by the sum of abdominal rumbling, cramps, flatulence, diarrhea, and vomiting (31).

Study Treatments

Whole sheep milk powder was sourced from Blue River Dairy (batch no. F2125/HC08) and Spring Sheep Milk Company (batch no. MAN: NOV17-JAN18) and these were blended 1:1 prior to weighing and reconstitution for both consumption and all subsequent milk analysis. Whole cow milk powder was commercially sourced from NZMP (New Zealand Milk Products, Fonterra Co-Operative Group, Auckland, New Zealand). Milk powders were stored frozen (−20°C) prior to use.

TABLE 1 | Proximate composition of 650 mL of the sheep and cow milk test drinks.

Component	Cow milk		Sheep milk	
	Amount	%	Amount	%
Total energy (kJ)	1649.3	-	2140.4	-
Fat (g)	21.3	3.3	33.4	5.1
Protein (g)	19.4	3.0	29.9	4.6
Lactose (g)	33.3	5.1	24.9	3.8
Total solids (g)	79.0	12.2	91.7	14.1
Solids non-fat (g)	57.7	8.9	60.3	9.3

Test drinks were prepared from 25 g whole cow milk powder or 30 g whole sheep milk powder reconstituted in 180 mL water, respectively, per a total reconstituted volume of 200 mL.

Test drinks made from reconstituted whole cow (CM) and whole sheep milk (SM) powder were provided in 650 mL quantities. Milks were prepared from milk powder the evening prior to the visit using heated (30°C) filtered water, shaken vigorously, then stored overnight at 4°C. Pre-weighed portions of cow milk powder or sheep milk powder (81 or 98 g, respectively) were reconstituted in 585 mL water. The reconstitution was according to the manufacturer's instructions, providing a similar sensory profile between milks, and to approximate the proportional solids content in an equal volume of fresh liquid milk. Milks were prepared in opaque plastic drinkware and served chilled. No sensory masking of products was used.

The nutrient and total amino acid composition of each milk (cow and blended sheep milk, respectively) are provided in **Tables 1**, **2**, respectively. Reconstituted SM was higher in total energy, fat, protein, total solids, and solids non-fat, but slightly lower in lactose than CM. With a higher total protein content than CM, the total amino acid content of SM was also greater. Proportions of amino acids as % content were generally similar (within 5%), aside from tryptophan and alanine which are present in 18 and 10% higher proportion in SM compared to CM, respectively.

Analysis Methodology

Chemicals

Sodium acetate trihydrate (pro analysis), acetic acid (glacial), acetonitrile (HPLC grade), and methanol (HPLC grade) were purchased from Merck KGaA (Darmstadt, Germany). Disodium hydrogen orthophosphate heptahydrate (98.0–102.0%), ethylenediamine tetraacetic acid (EDTA) (>99%), and orthophosphoric acid (>85%) were purchased from VWR Chemicals BDH (Radnor, PA, USA). Triethylamine (>99%), DL-norleucine (98%), L-asparagine (>98%), L-glutamine (>99%), L-tryptophan (>98%), DL-dithiothreitol (>985), and phenylisothiocyanate (>99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

The following reference materials were purchased from Sigma-Aldrich: Amino acid standard solution AAS18 (2.5 μmol mL^{-1} of 18 proteinogenic amino acids in 0.1 N HCl), Amino acid standard solution A6407 (2.5 μmol mL^{-1} of 26 physiological

TABLE 2 | Amino acid content of sheep and cow milk.

Amino Acid	Cow milk		Sheep milk	
	mg·mL^{-1}	% of total amino acids	mg·mL^{-1}	% of total amino acids
ESSENTIAL AMINO ACIDS				
Leucine	2.95	9.35	4.85	9.59
Lysine	2.50	7.92	4.12	8.15
Valine	1.94	6.14	3.17	6.26
Isoleucine	1.59	5.03	2.48	4.90
Phenylalanine	1.45	4.61	2.30	4.55
Threonine	1.40	4.43	2.25	4.44
Histidine	0.84	2.66	1.35	2.66
Methionine	0.67	2.12	1.13	2.22
Tryptophan	0.41	1.29	0.78	1.53
NON-ESSENTIAL AMINO ACIDS				
Glutamic acid†	6.32	20.02	9.64	19.04
Proline	3.04	9.63	4.90	9.68
Aspartic acid†	2.39	7.57	4.06	8.03
Serine	1.73	5.49	2.54	5.02
Tyrosine	1.41	4.45	2.24	4.42
Alanine	1.06	3.34	1.86	3.68
Arginine	1.06	3.37	1.61	3.18
Glycine	0.59	1.86	0.96	1.90
Cystine	0.23	0.72	0.37	0.73

† *Results for aspartic acid and glutamic acid may include contributions of asparagine and glutamine, respectively, converted during hydrolysis.*

amino acids in 0.1 N HCl), Amino acid standard solution A6282 (2.5 μmol mL^{-1} of 14 physiological, basic amino acids in 0.1 N HCl).

A proteinogenic amino acid standard consisting of amino acid standard solution AAS18 and 0.05 μmol mL^{-1} of L-asparagine, L-glutamine and L-tryptophan was prepared. A composite physiological amino acid standard was prepared from equal volumes of amino acid standard solutions A6407 and A6282.

Biochemical Analysis

Plasma free amino acids were analyzed by the AgResearch Analytical Laboratory (Palmerston North, New Zealand) using the Pico-Tag method (33). Each plasma sample (500 μL) was mixed with 25 μL of 80 mmol L^{-1} dithiothreitol in 200 mmol L^{-1} phosphate buffer as an antioxidant, and 10 μL of 10 mmol L^{-1} norleucine as an internal standard. The mixture was filtered through a 2-mL Vivaspin 500 centrifugal concentrator (10 kDa MWCO, Bio-Strategy, Auckland, NZ) and centrifuged (90 min, 10,000 x *g*, 4°C). The filtrate (50 μL) was lyophilized for 1 h in a freeze drier (Flexi-Dry, FTS Systems, Stone Ridge, NY, USA) and then reconstituted in 20 μL freshly prepared methanol/1 M sodium acetate/triethylamine (2:2:1) and lyophilized again (16 h). The dried sample was reconstituted in 20 μL derivatization solution (freshly prepared methanol/Milli-Q water/triethylamine/phenylisothiocyanate, 7:1:1:1), and incubated at room temperature for 20 min and then lyophilized (2 h). The dried sample was finally reconstituted in 200 μL of the sample diluent (0.71 mg mL^{-1} disodium hydrogen orthophosphate in Milli-Q water, pH adjusted to 7.40 with 10% phosphoric acid, diluted to 5% v/v in acetonitrile). The sample was centrifuged (5 min, 12,000 x *g*, room temperature), and the supernatant was used for the HPLC analysis.

Amino acids were resolved on a PicoTag® For Free Amino Acid Analysis column (60 Å, 4 μm, 300 mm x 3.9 mm) (Waters Corporation, Massachusetts, USA) using a LC-10ADvp instrument (Shimadzu Corporation, Kyoto, Japan). The extract 50 μL was injected onto the column, which was held at 46°C, and eluted over a 90-min gradient with a flow rate of 1 mL·min^{-1}. UV detection was carried out at 254 nm. The mobile phase was a mixture of buffers A and B. Buffer A consisted of 1,964 g stock buffer (38.16 g sodium acetate trihydrate dissolved in Milli-Q water and made up to 4 L, pH adjusted to 6.50 with 10% v/v acetic acid), 35 g acetonitrile, and 500 μL 10 mM EDTA. Buffer B consisted of 900 mL acetonitrile, 800 mL Milli-Q water, and 300 mL methanol. The gradient elution programme was as follows: held at 100% A (0–13.5 min); 0–2% B (13.5–24 min); 2–6% B (24–30 min); 6–28% B (30–50 min); 28–31.5% B (50–62 min); held at 31.5% B (62–70 min); 31.5–100% B (70–70.5 min); held at 100% B (70.5–74.5 min); 100–0% B (74.5–75 min); held at 100% A (75–90 min).

Analytical batches consisted of a blank, a derivatization blank, the proteinogenic amino acid standard, and the composite physiological amino acid standard, followed by up to eight samples injected sequentially. Standards were run after every eight sample injections. The derivatization blank was used to verify that the observed response of each analyte was not affected by the derivatization matrix. Quantification of amino acids was based on the mean standard response of each analyte. Typical chromatograms of human plasma are shown in **Supplemental Figure 1**.

Plasma glucose was measured using a Roche Cobas c311 by enzymatic colorimetric assay (Roche Diagnostics, Mannheim, Germany). Plasma insulin was measured using a Cobas e411 immunoassay analyser (Roche Diagnostics, Mannheim Germany).

Milk Compositional Analysis

Cow or sheep milk powders (12.5 and 15.0 g, respectively) were reconstituted in 90 mL MilliQ water for 20 min at room temperature. The sheep milk powder was blended 1:1 from the two suppliers prior to weighing and reconstitution as described above. The dissolved powders were then kept at 4°C overnight to reach full hydration. The samples were warmed to 40°C in a water bath in the following day. The proximate composition analysis was performed using a MilkoScan FT1 (FOSS, Denmark) analyzer using the default milk mosaic software. Amino acids content of the milk powders was determined by the AgResearch Analytical Laboratory (Palmerston North, New Zealand). Milk powders were hydrolysed using 7.5 M hydrochloric acid, performic acid oxidation, and alkaline hydrolysis with 4.67 M sodium hydroxide for acid stable amino acids, sulfur amino acids and tryptophan, respectively, prior to analysis. All amino acids were analyzed using a sodium-based ion exchange

TABLE 3 | Baseline participant characteristics.

Measure	Mean ± SEM
Age (years)	24.4 ± 1.1
BMI (kg/m^2)	23.3 ± 1.1
Waist circumference (cm)	77.2 ± 1.1
Glucose (mmol/L)	4.9 ± 0.1
Blood pressure (mmHg)	
Systolic	107 ± 3
Diastolic	72 ± 1

Values presented as means ± SEM from fasting samples collected on the first visit only, aside from glucose and blood pressure, which represent the mean of both visits.

chromatography with post-column derivatisation by ninhydrin with absorbance readings at 570 and 440 nm (modified AOAC methods 994.12, 994.12, and 988.15, respectively).

Statistical Analysis

A sample size of 30 subjects was determined to be required to detect a 20% difference in peak amino acid concentrations with a power of 90% using α 0.05. This was based on previously reported 60 min concentrations of 608 μmol·L^{-1} with a standard deviation of 198 μmol/·L^{-1} (34).

Statistical analyses were performed with SPSS version 25 (SPSS, IBM Corporation, Armonk, NY, USA). Continuous data are presented as mean ± SEM. Study outcomes were analyzed on a per protocol basis. Incremental area under the curve (iAUC) was calculated using the trapezoidal method, correcting for baseline concentrations. Outliers in amino acid data were identified as greater or <Q3+3IQR. Multiple imputation was used for values missing completely at random, as the mean of 5 iterations. Values lower than the limit of quantification were imputed at 50% of the limit of detection.

Continuous variables were analyzed using parametric tests. Single factor comparisons, such as iAUC, were made using Student's *t*-test with the null hypothesis that there is no difference between the test drink treatments. The null hypothesis was rejected, and the difference between treatments statistically significant, if $P < \alpha$ and $t > t_{crit}$. All outcomes with multiple factors were analyzed by repeated factor generalized linear model with milk and time compared within-subject and adjusted for multiple comparisons using a Sidak Holm adjustment. The Huynh-Feldt correction was used where Mauchly's sphericity test failed. Alpha was set at $P < 0.05$ for all tests.

Heat maps were created using R software version 2.15.2 (35) with gplots (heatmap.2), RColorBrewer and colorRamps packages (R Development Core Team).

RESULTS

Demographics

Thirty females completed the study. All subjects had anthropometric and biochemical values within a healthy range (**Table 3**).

Although all subjects indicated they "avoided drinking milk" (as a binary classification to determine eligibility), the majority of subjects reported avoiding dairy "most of the time" ($n = 14$; 47%) or "sometimes" ($n = 12$, 40%), with only four subjects (13%) indicating "seldom," and none (0%) responding "always." Similarly, the majority classified their restriction of dairy as "eat less than desired" ($n = 26$, 87%), with a minority indicating they "avoid altogether" or have "no restriction" ($n = 2$, 7% each).

Most subjects classified their self-reported symptoms from dairy as causing "occasional" ($n = 19$, 63%), or "usual" ($n = 9$, 30%) symptoms, with two subjects (7%) reporting "no problem." When screened for lactose intolerance, 24 (80%) subjects were identified as lactose intolerant (score >70), with a mean perceived symptom score of 168 ± 17 mm.

Fourteen subjects reported consuming a maximum of 250 mL milk per day in any form (i.e., including with tea, coffee, cereals, etc.; 47%), while the majority reported no daily consumption ($n = 16$, 53%).

Postprandial Amino Acid Response

Peak plasma BCAA concentrations (at 60 min) were greater following SM than CM ingestion (641.1 ± 16.3 vs. 563.5 ± 14.4 μmol/L; $p < 0.001$). Individually, all plasma BCAA responses differed between milks (**Figure 2**). Leucine and valine concentrations were higher following SM at all postprandial time points ($p = 0.001$ and $p < 0.001$ time × milk interaction, respectively; $p < 0.01$ for all postprandial time point pairwise comparisons; **Figures 2A,B**). Isoleucine concentrations were similarly higher following SM ($p = 0.031$ time × milk interaction), apparent postprandially but also at baseline ($p < 0.05$ all pairwise comparisons at 0, 60, 120, and 240 min; **Figure 2C**). Similarly, the iAUC for BCAAs in circulation was higher for SM than CM ($p < 0.05$; **Table 4**).

All individual amino acid responses are shown as a heatmap (**Figure 3**) displaying the percentage change from SM fasting concentrations (0 min). All amino acid concentrations changed over time independent of the type of milk consumed (**Supplemental Figures 2–4**; $p < 0.05$ each, respectively), except for taurine which did not change after milk ingestion ($p > 0.05$ main time effect). The majority increased with milk ingestion, while decreases were observed for tryptophan, cystine, glycine, and all non-proteogenic AA except ornithine and α-aminobutyric acid.

Aside from BCAAs, only the EAAs lysine and methionine, and NEAAs proline and alanine responded differently between milks ($p = 0.011$, 0.017, $p = 0.002$, and $p = 0.001$ time × milk interaction, respectively). Both lysine and methionine concentrations were higher at 240 min following SM than CM ($p < 0.05$ between milks; **Figures 2D,E**), with lysine concentrations additionally greater at the 60 min peak concentration ($p = 0.001$). Both lysine and methionine iAUCs were similarly greater with SM than CM ($p < 0.01$; **Table 4**). Tryptophan concentrations were higher with SM but were independent of postprandial time ($p = 0.012$ main milk effect; **Figure 2F**). Proline concentrations were higher following SM than CM at 240 min ($p = 0.016$; **Figure 2G**), which was mirrored in the iAUC ($p = 0.003$). Alanine concentrations

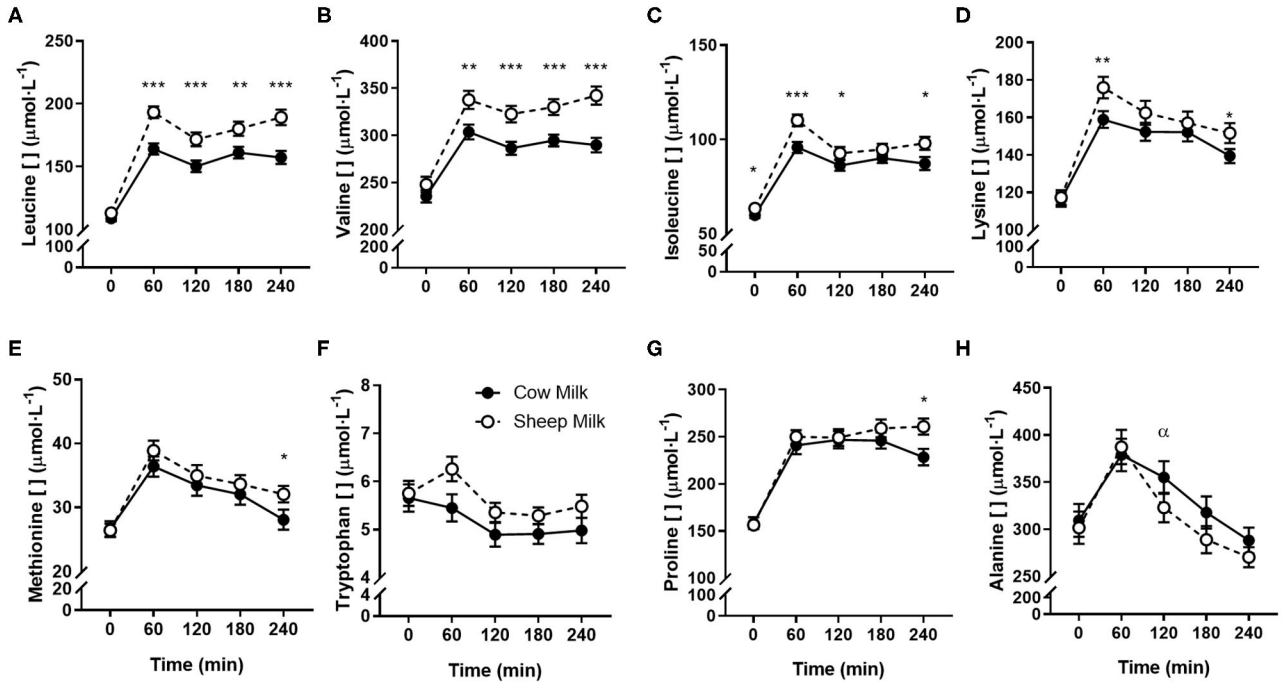

FIGURE 2 | Postprandial changes in plasma amino acids differing between sheep and cow milk following ingestion. Values presented as means ± SEM for leucine **(A)**, valine **(B)**, isoleucine **(C)**, lysine **(D)**, methionine **(E)**, tryptophan **(F)**, proline **(G)**, and alanine **(H)**. There was a main effect of milk for tryptophan ($p = 0.012$) and a time × milk interaction for all other presented amino acids ($p < 0.05$ each, respectively). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ denote statistical significance between sheep (○) and cow (●) milk; α denotes significant changes from baseline after cow milk (Sidak corrected *post hocs*).

did not differ between milks at any time point (**Figure 2H**), as reflected by similar iAUCs ($p = 0.305$), but the rise in alanine concentrations from baseline to 120 min was sustained following CM ingestion ($p = 0.004$) but not SM ($p > 0.05$). Although the iAUC for aspartic acid concentrations was greater for SM than CM ($p = 0.024$), this was not reflected as an interaction of milk and time ($p = 0.186$).

Postprandial Glycaemic Response

Following milk ingestion, plasma glucose responses to SM and CM were different (time x milk p = 0.002; **Figure 4**); however, the iAUC did not differ ($p = 0.145$). SM did not impact postprandial glycaemia, but CM resulted in decreased glucose concentrations from fasting to 45, 60, and 90 min ($p < 0.05$ for each) which were lower than those experienced with SM at 60 and 75 min ($p < 0.05$ for each), followed by a return to fasting concentrations at 240 min. In contrast, no differences in insulin response were observed (time x milk $p > 0.05$).

DISCUSSION

Milk is an important source of dietary protein and minerals, including calcium. However, avoidance of bovine dairy is increasingly frequent, requiring the identification of suitable dietary alternatives to support health. The composition of milk, both with respect to nutrient composition and protein structure, can influence digestive processes and appearance of nutrients in circulation. Despite well-known compositional differences between ruminant milks, including sheep and cow milks, few studies have comparatively described the postprandial impacts on nutrient appearance. The current study addressed this question by comparing the circulating amino acid postprandial responses, following ingestion of equal volumes of SM or CM. The composition of the milks differed, with SM containing a greater quantity of BCAAs and all other AA due to its higher protein content. In response to a single ingestion, there was a greater abundance of BCAAs, methionine, lysine, and proline in circulation. Yet, despite greater compositional abundance of all AAs, no greater circulating abundance of any of the other AAs were observed relative to CM, and in the case of alanine, plasma concentrations were higher after CM.

In the current study, female volunteers consumed a single bolus of 650 mL of either SM or CM. Plasma BCAA concentrations were elevated to a greater degree following SM consumption than CM, notably at peak concentrations (60 min). Peak BCAA concentrations, in particular leucine, have been hypothesized to be important in triggering muscle protein synthesis (MPS) (36–38). From 60 to 240 min, there continued to be higher plasma leucine concentrations following SM ingestion. In addition to the greater abundance of these BCAAs in SM from an equal volume, the higher concentrations in circulation may reflect differences in protein structure. For caseins, sheep milk AA sequences include notable differences to cow milk sequences across α_{s1}-, α_{s2}-, β–, and κ-caseins, which

TABLE 4 | Amino acid iAUC following sheep and cow milk.

Amino acid[a]	Cow milk	Sheep milk	*p* value[b]
Isoleucine	6,367 ± 336	7,465 ± 501	0.012
Leucine	10,417 ± 572	14,627 ± 901	<0.001
Valine	12,307 ± 702	17,606 ± 1,225	<0.001
Lysine	7,604 ± 605	9,652 ± 705	0.008
Methionine	1,361 ± 144	1,860 ± 170	<0.001
Threonine	3,520 ± 441	4,602 ± 650	0.073
Phenylalanine	1,713 ± 157	1,946 ± 187	0.140
Histidine	1,658 ± 257	1,958 ± 279	0.423
Tryptophan	−124 ± 43	−29 ± 45	0.076
Proline	17,639 ± 891	20,476 ± 1,087	0.003
Glutamic acid	1,309 ± 462	2,025 ± 319	0.055
Aspartic acid	55 ± 53	198 ± 41	0.024
Tyrosine	3,991 ± 213	4,097 ± 322	0.733
Asparagine	2,065 ± 185	2,340 ± 253	0.220
Glutamine	14,304 ± 1,700	13,122 ± 1,585	0.536
Serine	2,754 ± 321	2,519 ± 371	0.499
Arginine	1,541 ± 238	1,824 ± 220	0.195
Alanine	6,760 ± 2,046	4,716 ± 1,828	0.305
Cystine	−29 ± 12	−13 ± 10	0.243
Glycine	−3,241 ± 654	−4,768 ± 621	0.037[c]
Ornithine	1,922 ± 178	2,209 ± 206	0.169
Citrulline	−1,511 ± 112	−1,280 ± 120	0.124
Hydroxyproline	−91 ± 41	−126 ± 58	0.615
1-Methylhistidine	−132 ± 52	−270 ± 94	0.140
3-Methylhistidine	−82 ± 25	−38 ± 27	0.192
αAminobutyric acid	61 ± 244	85 ± 433	0.959
Taurine	−308 ± 328	51 ± 388	0.441

[a] *Values presented as means ± SEM in* $\mu mol \cdot L^{-1} \cdot min^{-1}$.
[b] *Significance analyzed by Student's t-test.*
[c] *Although* $p < 0.05$ *for Gly,* $t < t_{crit}$ *and the null hypothesis was not rejected.*

directly influence their enzymatic products and functionality including micelle formation and mineral binding (18). These factors, in addition to macronutrient interactions (including fat globule properties) and macrostructural effects (20), are known to influence digestion rates and AA appearance (20, 39). Irrespective of the underlying reason for higher circulating BCAAs following ingestion with SM, portion-for-portion SM may help to supply sufficient AA acutely and longer term. Indeed, in a rodent model, a similar elevation of leucine, along with lysine and methionine were seen in circulation following long term feeding with SM relative to CM (26). For some populations, such as the elderly, greater acute availability of AAs during peak periods, and also sustained concentrations in circulation following ingestion, may be beneficial for stimulation of MPS (40, 41). Pulse feeding of protein has been shown to improve protein retention in elderly women (40), while the delayed MPS observed following exercise in the elderly (41) may benefit from sustained availability of AAs. Future research should aim to identify whether clinical outcomes relating to elevated AA availability, including measures of MPS, are affected differently with SM ingestion.

Other essential (lysine and methionine) and non-essential (proline) AA concentrations were also elevated more following SM ingestion than CM. While this reflects the greater abundance of these AAs and total protein in SM, the implications of greater postprandial concentrations are less clear than for BCAAs. Indeed, greater intake of specific AAs have been shown to enrich circulating concentrations of these AAs during periods of growth (42). As with BCAAs, adequate postprandial abundance of EAAs during activation of MPS are required for its optimal stimulation (43), but under conditions of adequate abundance, greater postprandial elevations of specific AAs have not been clearly linked to greater MPS stimulation (43). Similarly, for long term health, greater bioavailability of EAAs such as lysine or methionine may be most relevant where these AAs are limited in the diet. Both lysine and methionine are found in short supply in cereal based foods (44), and can be limiting AAs in the diets of populations consuming largely cereal based diets, such as vegetarians (44). When limited in the diet, additional intake of lysine has been shown to have beneficial impacts on anxiety and stress (45), diarrhea (46), or even muscle strength and function in elderly women (47). Hence, in cases of insufficient dietary intake, as in those consuming greater proportions of cereals such as young children (48–51), SM could be an attractive nutrient dense option.

Although most AAs were more abundant in SM than CM, most did not appear in greater quantities in circulation. In the case of alanine, plasma concentrations were actually slightly lower following SM than CM, despite SM providing more alanine. Many ingested AAs are taken up by first pass metabolism by the splanchnic tissues (52), which could explain a lack of difference in circulation for AAs such as glutamine (53) or tryptophan (54), among other AAs (55, 56). Thus, any possible benefit of greater acute intake of these AAs abundant in SM may only be detectable within the gut or liver (27) and may not be apparent in peripheral circulation. Peripheral circulating AAs, in addition to influence from endogenous AA pools (52), are also impacted by gastrointestinal transit (57, 58), or postprandial metabolic responses to meals (58–60). Importantly, the current study did not use the gold standard techniques of isotopically labeled foods to precisely track the fate of all ingested AAs, which limits the current interpretation of the AA appearance differences. Differences in protein peptide structure (18) or physiochemical properties (16, 61) between SM and CM have the potential to influence micelle (62) or curd formation (63), gastric emptying (22), incretin responses (64, 65), or AA accretion independent of total AA content (23). While gastric emptying may impact AA appearance (57, 58), physiochemical properties like curd formation have not been conclusively shown to influence gastrointestinal transit (66–68). Further, comparative descriptions of such properties between SM and CM are limited (63). As such, more detailed descriptions of the physiochemical differences between SM and CM, and their respective influence on digestion dynamics, would be required to attribute circulating AA differences to physical influences of protein structures. Although insulin responses were similar between SM and CM, glucose concentrations decreased slightly with CM but not SM. While this could suggest differences in incretin response (69),

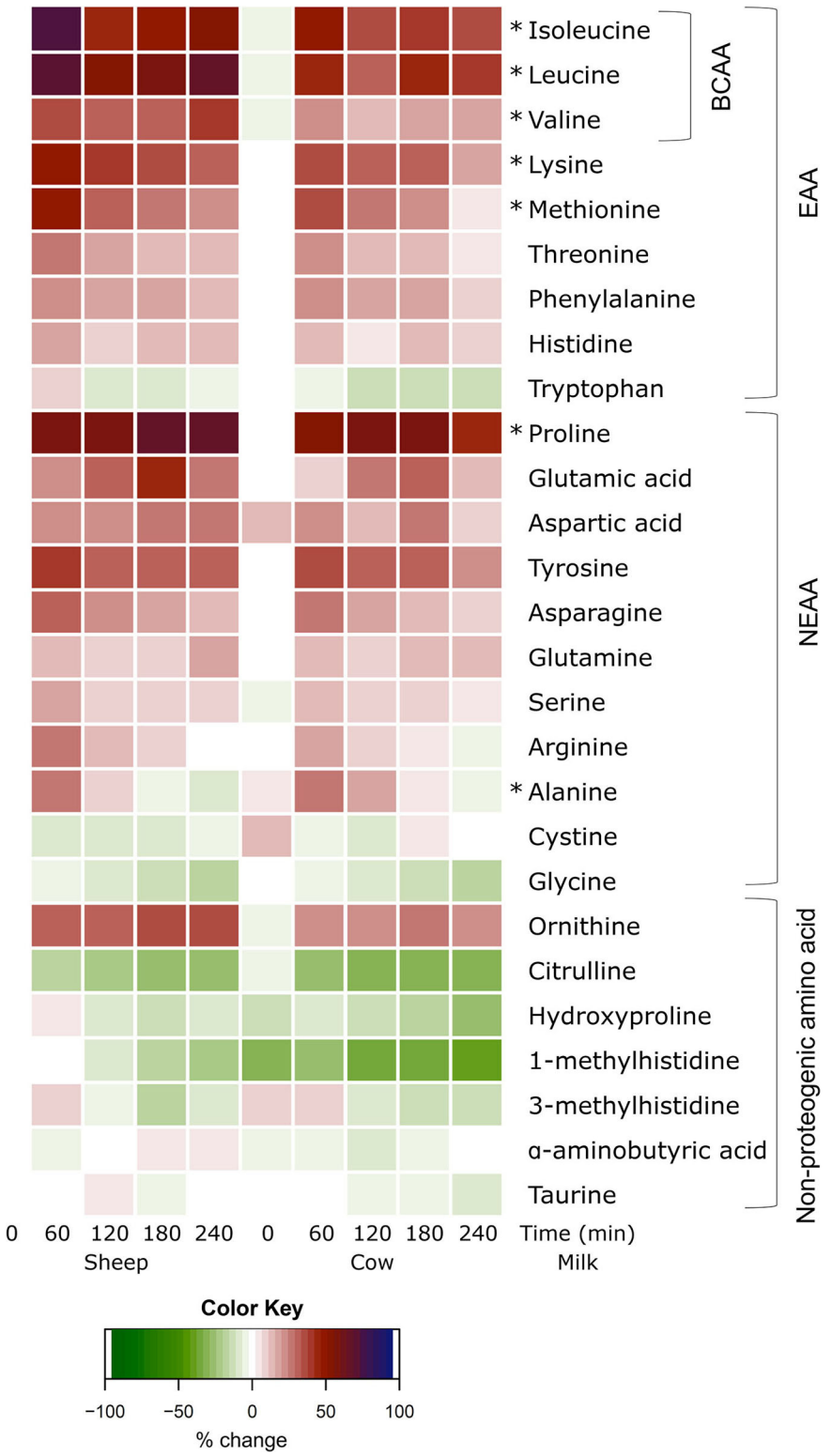

FIGURE 3 | Heat map of postprandial changes in individual plasma amino acids after sheep and cow milk ingestion. Values are presented as mean percent changes relative to concentrations at fasting prior to sheep milk ingestion. White represents a 0% change from sheep milk fasting concentrations. Red represents a 50% increase; blue represents a 100% increase; green represents a decrease. "0": timepoint 0 min; "60": timepoint 60 min; "120": timepoint 120 min; "180": timepoint 180 min; "240": timepoint 240 min. Amino acids are grouped descending from the top by branched-chain amino acids (BCAA), essential amino acids (EAA), non-essential amino acids (NEAA), and non-proteogenic amino acids. *denotes significant ($p < 0.05$) interaction of time × milk.

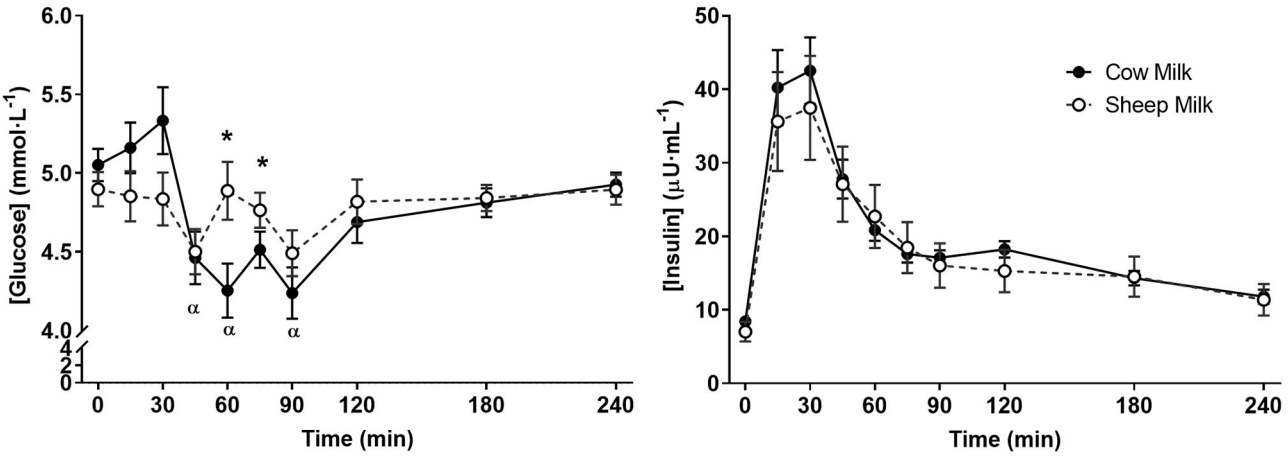

FIGURE 4 | Postprandial concentrations of glucose and insulin following sheep and cow milk. Values represent means ± SEM in mmol·L^{-1} for glucose and μU·mL^{-1} for insulin. There was a time × milk interaction for glucose but not insulin ($p < 0.05$ and $p > 0.05$, respectively). *$p < 0.05$ denotes statistical significance between sheep (○) and cow (•) milk; α denotes significant changes from baseline after cow milk (Sidak corrected *post hocs*).

gastrointestinal motility or appetite hormone responses were not addressed in this study. Hence, further work is required to understand the specific influence of protein and peptide differences on aspects of digestion and postprandial metabolism impacting circulating AAs.

Comparative assessment of meals with inherently different composition is challenging, as differences may exist not only in the primary nutrient of interest, but across other macro and micronutrients, including total energy. In the current study, equal volumes of milk were selected to provide a comparison of sheep and cow milk on a "portion-for-portion" basis, resulting in greater total protein and specific AA content in SM relative to CM. Although this compositional difference contributes to the greater postprandial rises in AAs observed following SM, it is important to note that fat interactions (70, 71), total energy (72, 73), and volume (74) also impact digestion and possibly nutrient appearance (58, 75) in circulation. Artificially matched formulations to provide equivalent protein and/or AA content would similarly alter or eliminate other compositional differences inherent between milks, which contribute to the overall postprandial response. However, it should also be noted that the SM used in this study had slightly lower total solids content than what has previously been reported for liquid milk from New Zealand herds (27). The lower total solids concentration (∼80% of liquid sheep milk) was due to the reconstitution protocol used, which was according to the manufacturers' instructions. While the use of processed milk may have also impacted compositional features of the milks, including peptide structures, physiochemical properties, and digestion kinetics (20), there exist few comparisons of fresh pasteurized and powdered milk to inform how this processing technique (39) (particularly in the context of sheep milk) may have impacted the current findings. Thus, this study provides insight into the post-meal AA response to an equal serve of powdered SM relative to CM, but may not be predictive of differences in the digestion and postprandial AA appearance of sheep milk proteins if provided in quantities matched to cow milk, other regional variations in proportions, or differing processing techniques.

Although the current findings are a useful description of typical AA responses to differing ruminant species milks, it is important to note that the postprandial dynamics were described only in females, and further in dairy avoiders who generally restrict dairy consumption. These subjects, although self-described as avoiders, were largely lactose intolerant, yet many did consume some milk, albeit infrequently and in small amounts. Partial rather than complete avoidance has similarly been reported as more likely in Australian symptomatic dairy avoiders (1). This restriction of dairy, paired with intolerance, may have impacts on gastrointestinal transit and overall tolerance of milk, as habitual dairy consumption in lactose intolerance has been known to reduce malabsorption and intolerance (76, 77), including symptoms of flatulence (77). However, AA appearance in circulation has been shown to be unimpaired in subjects with lactose intolerance (78), although other forms of dairy intolerance may impact AA (78) and other nutrient appearance (79). While variation in dairy intake in the habitual diet may be expected to impact fasting amino acid profiles, we have previously demonstrated that changes to habitual dairy intake, such as restriction or increased intake over 1 month does not alter BCAA profiles, or indeed any other AA, in circulation (80). Yet, subjects who restricted dairy in the study by Prodhan et al. (80) still consumed 1.2 servings of dairy per day; as the current study did not record habitual dairy intake by means of a food frequency questionnaire or dietary records, it is unknown whether these participants had habitual intakes even below those previously reported. Indeed, others have reported that habitual protein intake, such as a high protein diet over 1 week, influences the postprandial AA response including N retention, albeit this effect has been shown to be more pronounced with soy intake rather than dairy (81). Hence, while the population studied here

may have had alterations in habitual dairy intake or symptoms experienced with dairy relative to habitual consumers, it is less likely that this influenced postprandial AA appearance.

In summary, ingestion of SM and CM results in different responses when compared "portion-for-portion." SM results in a greater circulating increase in the BCAAs leucine, isoleucine and valine, as well as the EAAs lysine and methionine and the non-essential AA proline compared to CM. These differences are not just a result of the compositional differences between SM and CM, but also reflect the inherent differences in protein digestion and amino acid absorption. The greater uptake of BCAAs may make SM an attractive, nutrient-dense alternative for consumers seeking bovine milk alternatives, including those who habitually avoid milk or have higher muscle maintenance requirements such as young children, the elderly and athletes. In addition, the greater uptake of lysine and methionine with SM may be beneficial for consumers of a vegetarian diet where these amino acids can be limiting.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because data described in the article, code book, and analytic code will not be made available because approval has not been granted by subjects. Requests to access the datasets should be directed to David Cameron-Smith, d.cameron-smith@auckland.ac.nz.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by New Zealand Health and Disability Ethics Committees (Reference no. 18/NTB/92). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AM designed and conducted research, analyzed data and performed statistical analysis, and wrote the paper. LS designed research, performed statistical analysis, and wrote the paper. AS conducted research, analyzed data, and wrote the paper. PS conducted research. LD designed research and wrote the paper. DC-S designed research and had primary responsibility for final content. All authors approved the final version of the manuscript for submission. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2020.553674/full#supplementary-material

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Bifidobacterium infantis Metabolizes 2′Fucosyllactose-Derived and Free Fucose Through a Common Catabolic Pathway Resulting in 1,2-Propanediol Secretion

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Human milk oligosaccharides (HMOs) enrich beneficial bifidobacteria in the infant gut microbiome which produce molecules that impact development and physiology. 2′fucosyllactose (2′FL) is a highly abundant fucosylated HMO which is utilized by *Bifidobacterium longum* subsp. *infantis*, despite limited scientific understanding of the underlying mechanism. Moreover, there is not a current consensus on whether free fucose could be metabolized when not incorporated in a larger oligosaccharide structure. Based on metabolic and genomic analyses, we hypothesize that *B. infantis* catabolizes both free fucose and fucosyl oligosaccharide residues to produce 1,2-propanediol (1,2-PD). Accordingly, systems-level approaches including transcriptomics and proteomics support this metabolic path. Co-fermentation of fucose and limiting lactose or glucose was found to promote significantly higher biomass and 1,2-PD concentrations than individual substrates, suggesting a synergistic effect. In addition, and during growth on 2′FL, *B. infantis* achieves significantly higher biomass corresponding to increased 1,2-PD. These findings support a singular fucose catabolic pathway in *B. infantis* that is active on both free and HMO-derived fucose and intimately linked with central metabolism. The impact of fucose and 2′FL metabolism on *B. infantis* physiology provides insight into the role of fucosylated HMOs in influencing host- and microbe-microbe interactions within the infant gut microbiome.

Keywords: bifidobacteria, human milk oligosaccharides, fucose, 2′fucosyllactose, microbiota, microbiome

INTRODUCTION

Human milk nourishes infants and contains host-indigestible human milk oligosaccharides (HMOs) in addition to bioavailable nutrients. These compounds contribute to overall infant nutrition by shifting gut microbiome composition to favor beneficial microbiota that metabolize HMOs to bioactive products (1–5). Despite incorporating the same monosaccharide building blocks, HMOs vary structurally at the level of primary sequence and are diversified by degree of polymerization, branching, and secondary modifications such as fucosylation (6). 2′ Fucosyllactose

(2′FL) is the most abundant fucosylated HMO and accounts for up to 30% of total HMOs in secretor women (6, 7). 2′FL has been designated generally recognized as safe by the U.S. Food and Drug Administration as well as obtaining similar status by other regulatory agencies. As a consequence, it is added to commercial infant formula to more closely mimic the composition of human milk.

Bifidobacterium longum subsp. *infantis* (*B. infantis*) is often the dominant microbe in the infant gut microbiome where it utilizes fucosylated HMOs such as 2′FL. Several studies report that *B. infantis* utilizes 2′FL as a sole carbohydrate source (8–11). The metabolic fate of the fucosyl moiety of 2′FL, however, remains uncertain in its intracellular catabolism. Mechanistic hypotheses based solely on genomics are hindered due to the absence of identifiable fucose-active genes. Specifically, *B. infantis* lacks the full complement of the canonical pathway genes possessed by other bacteria such as *Escherichia coli* (12–14). This catabolic pathway results in the production of 1,2-propanediol (1,2-PD) through a series of phosphorylated intermediates (15, 16). Briefly, the pathway is regulated by *fucR* and relies on a fucose permease (*fucP*) to import fucose intracellularly where it is subsequently catabolized. Fucose isomerase (*fucI*) converts L-fucose to L-fuculose, which is subsequently phosphorylated by fucose kinase (*fucK*). L-fuconate-1-phosphate is converted to dihydroxyacetone phosphate and lactaldehyde by an aldolase (*fucA*). Dihydroxyacetone phosphate can subsequently be shunted to the central glycolytic pathway and lactaldehyde converted to 1,2-PD by an oxidoreductase (*fucO*) under anaerobic conditions (17). *B. infantis* ATCC 15697^{T} lacks the majority of homologous genes predicted in this pathway, although the genome encodes *fucO* which could produce 1,2-PD from lactaldehyde. Despite this, *B. infantis* has been previously observed to metabolize free fucose and produces 1,2-PD in co-fermentation with a low concentration of glucose (14).

Other bacteria such as *Xanthomonas campestris* and *Campylobacter jejuni* catabolize fucose through phosphorylation-independent pathways to produce lactate as an end product rather than 1,2-PD (12, 13) Moreover, *Sphingomonas* sp. metabolizes rhamnose, an isomer of fucose, to produce lactadehyde and pyruvate (18). The pathway requires a permease prior to intracellular conversion of fucose to fuconolactone by fucose dehydrogenase, and then hydrolyzed to form fuconate by fuconolactone hydrolase. Fuconate dehydratase then catalyzes the production of 2-keto-3-deoxy-fuconate, which is further dehydrogenated to 2,4-diketo-3-deoxy-fuconate. The metabolic fate of 2,4-diketo-3-deoxy-fuconate diverges in that *X. campestris* produces lactate and pyruvate by a hydrolysis reaction whereas *Sphingomonas* sp. produces lactaldehyde and pyruvate by an aldol reaction. Homologous genes to those used in these pathways are identifiable in the *B. infantis* genome. The presence of these putative fucose genes informed a hypothetical bifidobacterial fucose metabolic pathway in *B. infantis* and other infant-associated bifidobacteria (11, 14).

Studies of bifidobacterial fucose metabolism have generally focused on fucosylated HMOs such as 2′FL. Identification of homologous genes, functional genomic analyses such as transcriptomics, and quantitation of metabolic end products 1,2-PD and short-chain fatty acids have heretofore provided the primary evidence for the bifidobacterial fucose metabolic pathway (11, 14, 19). Thus the mechanism underlying bifidobacterial fucose metabolism remains hypothetical in the absence of direct observation of metabolic intermediates and loss-of-function genetic perturbations. Furthermore, *B. infantis* growth on free fucose as a sole carbohydrate source is incompletely characterized as existing studies focus on demonstrating *B. infantis* growth on free fucose or in co-fermentation (14, 20–22). As such, much uncertainty remains in the path by which *B. infantis* utilizes fucose as a free product or integrated into HMOs.

This study investigates *B. infantis* utilization of 2′FL-derived and free fucose as well as the impact of carbohydrate source and concentration on fucose metabolism. In order to explicate *B. infantis* physiology while metabolizing fucose, our experimental system quantifies growth kinetics, secreted end products, the proteome, and transcriptome. As a consequence, an integrated systems-level resolution of *B. infantis* utilization of 2′FL and fucose is presented herein.

MATERIALS AND METHODS

Bacterial Strains and Propagation

B. longum subsp. *infantis* strain ATCC 15697^{T} (originally isolated from human infant feces) was used in this study. Propagation was performed in de Mann, Rogosa and Sharpe (MRS; BD Difco, USA) supplemented with 0.05% (wt/vol) L-cysteine hydrochloride (Sigma-Aldrich, USA) at 37°C under anaerobic conditions (7% H_2, 10% CO_2, N_2 balance) (Coy Lab, USA).

Microplate Growth Assay

Growth phenotypes were analyzed in a 96-well format using a PowerWave HT Microplate Spectrophotometer (BioTek, USA). Overnight cultures were inoculated 1% (vol/vol) to modified MRS media (mMRS) that contains a defined carbohydrate substrate without acetate and Tween80. Carbohydrates include glucose, lactose, galactose, (Sigma-Aldrich, USA), fucose (Chem-Impex, USA), 2′fucosyllactose (BASF, Germany), and mixtures thereof at varying final concentrations (wt/vol) as the sole carbohydrate source(s). The growth assay was conducted anaerobically at 37°C until bacterial growth as measured by optical density at 600 nm (OD_{600}) reached stationary phase. Each carbohydrate source or mixture of sources were evaluated in biological triplicates with three technical replicates. Inoculated mMRS without carbohydrate served as the negative control, and inoculated mMRS with 2% (wt/vol) lactose served as the positive control. Bacterial growth kinetics were calculated using Wolfram Mathematica with the equation described by Dai et al., $\Delta OD\,(t) = \Delta OD_{asym}\left\{\frac{1}{1+\exp\left[k(t_c-t)\right]} - \frac{1}{1+\exp\left[kt_c\right]}\right\}$ where ΔOD corresponds to the adjusted optical density, ΔOD_{asym} the optical density at stationary phase, k the growth rate, and t_c the time to reach maximum growth rate (23). The growth assay was performed for 72 h at 37°C under anaerobic conditions by shaking for 5 s and assessing OD_{600} at 15-min intervals with triplicate biological and technical replicates of each broth

composition. At critical points during bacterial fermentation, three technical replicates were selected at random and reserved for intracellular metabolite analysis.

Bacterial metabolism was quenched with a solution of 900 μL 60% methanol (Thermo Fisher Scientific, USA) with 0.65% (w/v) ammonium carbonate (Honeywell, USA) at −20°C added to 300 μL cell-free growth media (24, 25) The solution was thoroughly mixed and incubated 30 min at −20°C prior to pelleting cells and reserving supernatant. Cell pellet was washed with 900 μL methanol/ammonium carbonate solution at −20°C. Supernatant was reserved and diluted with 900 μL HPLC-grade water (Thermo Fisher Scientific, Waltham, MA) at 4°C. Supernatant solution was thoroughly mixed prior to storage at −80°C. Reserved pellets were resuspended in 500 μL methanol with 24 mg/L adonitol (Sigma-Aldrich, St. Louis, MO) and stored at −80°C.

Growth Inhibition Assay

Overnight cultures were inoculated 1% (vol/vol) to MRS supplemented with 0.05% (wt/vol) L-cysteine hydrochloride (Sigma-Aldrich, USA) modified with added 1,2-PD, lactate, or phosphate-buffered saline. Each condition was evaluated in biological triplicate. Lactate was used as a control as it is known to inhibit *B. infantis* growth. MRS and equal volumes of phosphate-buffered saline served as additional controls. 1,2-PD concentration ranged from 0.08 to 168 mM and lactate concentration ranged between 0.07 to 144 mM. Cultures were grown anaerobically at 37°C for 24 h after which bacterial growth was measured by OD_{600}. Inhibition was determined using a comparison of the final OD_{600} at each concentration as compared to other concentrations and controls.

Characterization of Microbial Metabolic Intermediate and End Products

Bacterial growth for metabolic profiling was performed in 15 replicates of 2 mL mMRS inoculated at a concentration of 1% (v/v) and incubated for 48 h at 37°C under anaerobic conditions. OD_{600} was assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). Harvested cells were pelleted, washed with 1.5 mL 1× phosphate-buffered saline (PBS), and resuspended in 1 mL RNAlater (Sigma-Aldrich, St. Louis, MO). Cells resuspended in RNAlater were incubated for 8 h at 4°C prior to storage at −80°C. Cell-free spent growth media (500 μL) was reserved and quenched as described above and stored at −80°C.

Intracellular metabolites were extracted and derivatized for GC-MS analysis. Suspended pellets were thawed to −20°C, transferred to 2 mL tubes containing 600 mg 100 μm diameter silica lysis matrix (OPS Diagnostics, USA), and subjected to bead beating (3 ×{40 s at 6.0 m/s followed by 5 min incubation at −20°C}) using a FastPrep 24 bead beater (MP Biomedicals, USA). Whole-cell lysate was transferred to a 2 mL Safe-Lock tube (Eppendorf, USA) and lysing matrix was washed with 500 μL methanol. 1 mL chloroform (Thermo Fisher Scientific, USA) was added to the pooled whole-cell lysate and methanol wash and vortexed 2 min. Samples were incubated 4 h at −20°C and vortexed for 1 min at 1-hour intervals. Debris was pelleted at 21,200 × g for 5 min in a 4°C rotor, supernatant was transferred to a clean 2 mL Safe-Lock tube and dried overnight using a Vacufuge (Eppendorf, USA) in V-AL mode at room temperature with rotation. Dried extracts were derivatized following a modified protocol (25, 26). 20 μL pyridine (Sigma-Aldrich, USA) with 20 mg/mL methoxyamine hydrochloride (Sigma-Aldrich, USA) was added to dried extracts and vortexed 2 min prior to 90 min incubation at 32°C with continuous mixing. Thirty-two μL *N*-methayl-*N*-(trimethylsilyl)trifluoroacetamide (Sigma-Aldrich, USA) was added and samples were vortexed 2 min prior to incubation (30 min at 37°C, 60 min at 32°C, and 60 min at room temperature) with constant mixing. Debris was pelleted at 17,000 × g for 5 min. 50 μL supernatant was transferred to a vial insert and stored at −80°C overnight prior to measurement. 24 mg/L standards (pyruvate, lactate, 1,2-propanediol, *L*-fucono-1,4-lactone) (Sigma-Aldrich, USA) in methanol were derivatized following the above protocol. GC-MS analysis was performed at the University of Massachusetts Mass Spectrometry Center using an Agilent 6890/5973 GC- MS (Agilent Technologies, USA). In summary, 1 μL of the derivatized sample was injected in split mode (10:1). Gas chromatography was performed on a 30 m, 0.25 mm, 0.25 μm film thickness DB-5ms column (J&W Scientific, USA) using 1.2 mL min^{-1} helium flow. Ionization of analytes was done by electron ionization (EI +) at 70 eV. After 2 min at 70°C, the temperature was increased to 200°C at 12°C s^{-1}, then to 250°C at 8°C s^{-1}, and to 325°C at 12°C s^{-1}, followed by an additional constant temperature period of 5 min at 325°C. The transfer line temperature was set to 290°C. Full scan mass spectra were obtained from 45 to 600 m/z with 2 scans s^{-1} and a solvent delay time of 4 min. Compounds were identified using Agilent MSD ChemStation Software (Version E02.02, Agilent Technologies, USA) and NIST Spectral Search Program with NIST Standard Reference Database (Version 2.2, NIST, USA).

Extracellular metabolites were quantitated by HPLC. One mL quenched cell-free spent growth media concentrated using a Vacufuge for 4 h in V-AL mode at room temperature with rotation. Dehydrated solutes were resuspended in 200 μL HPLC-grade water and filtered through a 0.22-μm filter (Sartorius, USA) and stored at −80°C until analysis. Metabolite (2′fucosyllactose, fucose, lactose, glucose, 1,2-propanediol, acetate, lactate, and formate) concentrations were quantified using an 1260 Infinity HPLC system (Agilent Technologies, USA) equipped with a Optilab T-rEX refractive-index detector (Wyatt Technology, USA). Separation was performed using an Aminex HPX-87H column (ID 7.8 mm by 300 mm; Bio-Rad Laboratories, USA) at 30°C in a mobile phase of 5 mM H_2SO_4 with a flow rate of 0.6 mL/min and an injection volume of 20 μL. Analytical standards of 2′FL (BASF, Germany), fucose, lactose, glucose, organic acids (Sigma-Aldrich, USA), and 1,2-propanediol (Alfa Aesar, USA) were used to generate a standard curve from seven concentrations. Each measurement was performed in duplicate.

Proteome Sample Preparation

Cultures were harvested at mid-exponential phase and the pellet was washed 3X with PBS buffer and stored at −80°C until subsequent analysis. Cell pellets were suspended in 1X SDS lysis

and solubilization buffer (Protifi LLC, USA) and were sonicated with Bioruptor pico (Diagenode, USA) at high power for 10–15 min (sonication cycle: 30 s ON, 30 s OFF). The cell lysate was clarified by centrifugation at 13,000 g for 8 min. Protein concentrations were measured using the Pierce BCA protein Assay kit (Thermo Fisher Scientific, USA) on a Nanodrop spectrophotometer. Sample preparation for mass spectrometry was performed using the S-Trap micro universal MS sample prep kit (Protifi LLC, USA). Samples were reduced, alkylated, and trypsin digested according to manufacturer instructions. Briefly, 30–40 μg protein was loaded on S-Trap micro spin columns and washed with wash buffer. Each sample was mixed with digestion buffer containing mass spectrometry grade trypsin (Promega, USA) at a 1:50 ratio (wt/wt) and was added directly to the micro spin column. Samples were incubated in a water bath at 47°C for 1 h followed by overnight incubation at 37°C. Peptides were eluted by serial additions of 50 mM TEAB, 0.2% formate, and 0.2% formate in 50% acetonitrile. Eluted peptides were pooled and evaporated in a Vacufuge to dehydration and stored at −80°C until subsequent analysis. Tryptic peptides were purified with ZIPTIP® C18 pipette tips (Merck Millipore, USA) as per manufacturer instructions prior to MS/MS analysis.

Mass Spectrometry Proteomics

Tryptic digests were analyzed using a Thermo Easy-nLC 1000 nanoLC system coupled to a Thermo Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, USA). Peptides were separated using a 90-min gradient of increasing concentration of acetonitrile and infused into the mass spectrometer at 300 nl/min. MS1 data was acquired at 60,000 resolution in the Orbitrap and MS/MS data were acquired in a data-dependent manner selecting the top five most intense ions for fragmentation, using a nominal CID collision energy of 35%. Data were processed using Proteome Discoverer 2.0 (Thermo Fisher Scientific, USA). MS/MS data was queried against a database constructed from the UniProt database (www.uniprot.org) containing *Bifidobacterium longum* subsp. *infantis* ATCC 15697 (https://www.uniprot.org/proteomes/UP000001360, Proteome ID: UP000001360). Proteomes were analyzed using SEQUEST (27). Proteome Discoverer 2.3.0.523 (Thermo Fisher Scientific, USA). SEQUEST was searched with a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 10.0 PPM. Carbamidomethyl of cysteine was specified as a fixed modification. Variable modifications were specified including methionine oxidation and acetylation of peptide n-termini. For protein identification and quantification, a cut-off value of at least one unique high confidence peptide per protein, corresponding to a 1% false discovery rate (FDR) at the peptide level and peptide rank 1 protein was selected. Proteins and peptides identified and quantified by at least two of three replicates were used for comparisons. Mass spectrometry data were obtained at the University of Massachusetts Amherst Mass Spectrometry Center. Proteome data is deposited in the EMBL-EBI PRoteomics IDEntifcations Database (PRIDE) under Accession: PXD021868.

Proteome Data Analysis

Scaffold version 4.9.0, (Proteome Software Inc., USA) was used to validate peptide and protein calls. Peptide identifications were accepted at >99.0% probability by the Peptide Prophet algorithm (28) with Scaffold delta-mass correction. Protein identifications were accepted at >99.0% probability and containing at least two identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm (29). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. In order to generate relative quantification across samples, result files from Proteome Discoverer were imported into Scaffold and sf3 files were exported for the qualitative and quantitative comparisons. Peptide and protein abundances were normalized based on total sum of peptide and protein abundances, respectively, and were log10 transformed prior to the analysis. Multivariate data analysis was carried out using MetaboAnalyst 4.0 (30) (www.metaboanalyst.ca). Partial Least Squares Discriminant Analysis (PLS-DA) was used to maximize the covariance between variables. Variable importance of projection (VIP) score plot obtained by PLS-DA identified proteins (VIP score of ≥1.0) responsible for the clustering of groups. Proteins were annotated with GO terms using UniProt ID mapping (www.uniprot.org) and KEGG metabolic pathways were reconstructed using KEGG Mapper. PLS-DA, VIP, and KEGG metabolic pathways are reported in **Supplementary Figures 1–3**. The obtained proteomes were queried for proteins involved in the hypothetical *B. infantis* fucose metabolic pathway based on putative fucose genes identified in the *B. infantis* genome.

RNA-seq Transcriptome Library Preparation

For RNA extraction, cells stored in RNAlater (MilliporeSigma, Burlington, MA) at −80°C were centrifuged at 14,000 × g for 3 min and cell pellets were washed with 1 mL cold 1X PBS buffer. Total RNA was extracted using Ambion RNAqeous kit (Life Technologies, USA) according to manufacturer instructions with a beadbeating step added to disrupt the cells. Accordingly, cells were suspended in 600 μL lysis buffer in beadbeating tubes (100 μm Silica Beads; OPS Diagnostics, USA) at 5.5 m/s for 30 sec 1X using a FastPrep 24 bead beader (MP Biomedicals, USA). RNA concentrations were measured by a NanoDrop spectrophotometer and samples were subjected to DNase treatment using the Ambion Turbo DNA-free kit (Invitrogen, USA) using 2 μL of DNase I for 45 min for 25 μL eluted total RNA. The RNA was evaluated for genomic DNA contamination using qRT-PCR performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, USA) as described previously (31). An additional round of DNase treatment was performed for samples with high levels of residual DNA.

DNase-treated RNA was quantified using the Qubit High Sensitivity RNA Assay Kit (Life Technologies, USA) and RNA quality was assessed through RNA integrity number equivalent (RIN^e) as determined on a TapeStation 2200 system using High Sensitivity RNA screen tapes and reagents (Agilent Technologies,

USA). Samples with RIN^e >7.0 were subjected to ribosomal RNA depletion and mRNA purification with the RiboMinus kit with Magnetic Bead Clean up Module using the custom Pan-Prokaryote Probe Mix to target bacterial rRNA (Thermo Fisher Scientific, USA). RNA was quantified using the Qubit High Sensitivity RNA Assay and rRNA depletion was confirmed on TapeStation 2200 system. The depleted RNAs (RIN^e ∼1.0–2.5) proceeded to sequencing library generation with the NEBNext Ultra II Directional Library preparation kit (New England Biolabs, USA). Purification was performed with AmPure XP beads (Beckman Coulter, USA) and indexed using NEBNext® Multiplex Oligos for Illumina Indices (Dual Index Primers Set 1; New England Biolabs Inc., USA) with 8–10 PCR cycles which varied according to input RNA concentration. The libraries were quantified using the Qubit double-stranded DNA (dsDNA) HS assay (Life Technologies, USA). The quality of library products was measured by high sensitivity DNA screen tapes on the TapeStation 2200 system. Sequencing libraries were pooled in equimolar concentrations (4 nM) and denatured immediately prior to sequencing following Illumina pooled library instructions. Sequencing was performed on an Illumina NextSeq platform (NextSeq 500/550 High Output Kit v2.5, paired-end, 150 cycles, 5% Phi-X) at the Genomics Resource Laboratory, University of Massachusetts Amherst. Raw reads are publicly deposited in the NCBI Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/) under accession number GSE159189.

Transcriptome Informatic and Statistical Analysis

Raw reads were uploaded to the Massachusetts Green High-Performance Computing Cluster used for all computational/statistical analyses unless specifically noted. Sequencing adaptors were trimmed during de-multiplexing. The reads were aligned to the reference *Bifidobacterium longum* subsp. *infantis* ATCC 15697 (NCBI accession number: NC 011593.1) using bowtie-2 (v2.3.4.3) (32). Total and non-unique gene reads aligning to a specific genomic locus, as well as calculated raw counts (HTSeq count, v0.10.0) (33) were obtained for differential expression analysis.

Differential Gene Expression

In order to identify and quantify the magnitude of differentially expressed genes, the R package DESeq2 was used to analyze the raw count data (34). Genes with a sum count <50 were removed from analysis by pre-filtering. DESeq2 applies the Wald test for statistical analysis with adjusted $p \leq 0.05$ designated as statistically significant. Principal component analysis (PCA) was performed and plots were visualized using DESeq2 (34). Gene maps were created and visualized using the R package genoplotR following *B. infantis* ATCC 15697 reference genome obtained from NCBI (NC_011593.1) and colored with corresponding z–score (35, 36). Z–score for each gene of interest was calculated using the following equation:

$$\text{z} - \text{score} = \frac{Expression - Mean\ expression\ across\ all\ samples}{Standard\ deviation}$$

where expression corresponds to the normalized counts of the gene of interest. Z-scores were visualized using a heatmap or gene map. Volcano plots were created and visualized using the R package EnhancedVolcano (37). Predicted gene functions were determined using a combination of BLAST (38) and KEGG (http://www.genome.jp/kegg~searches).

Statistical Analyses

Statistical analysis of growth kinetics was performed using the final OD_{600} as a measure of total biomass. Final OD_{600} was subjected to one-way analysis of variance and Tukey's HSD for multiple comparisons between carbohydrate source. Metabolite concentrations were subjected to one-way analysis of variance and Tukey's HSD test for multiple comparisons between carbohydrate source. Fold change in gene expression was subjected to the Wald test during processing in DESeq2 (34). Z-scores generated from normalized counts were subjected to one-way analysis of variance and Tukey's HSD test for multiple comparisons between 2′FL concentrations and carbohydrate source.

Experimental Layout

Co-fermentation of free fucose with glucose and lactose in both large volume (5 mL) and standard 96-well microplate formats was performed. Large volume cultures were used to obtain sufficient biomass to perform intra- and extracellular metabolite assays as a function of growth time during co-fermentation of free fucose and glucose as well as during growth on 2′FL as a sole carbohydrate. Proteomic and transcriptomic analyses were performed using large volume cultures in order to obtain sufficient biomass to extract the necessary quantities of protein and RNA. 96-well microplate growth assays were performed in all other cases where growth kinetics and metabolite production were examined. A brief schematic of the experimental layout is included in **Supplementary Figure 4**.

RESULTS AND DISCUSSION

B. infantis Utilizes Free Fucose as a Carbohydrate Source

In order to understand *B. infantis* metabolism of free fucose and HMO-derived fucose, *B. infantis* ATCC 15697 was subjected to growth on fucose as a sole carbohydrate source and in co-fermentation with other sugars. Co-fermentation was performed due to prior observation of inconsistent *B. infantis* growth on fucose as the sole carbohydrate source which increased with the addition of trace glucose (14, 20, 21). Growth was performed in a standard 96-well plate format to maximize sensitivity and throughput and percentage carbohydrate substrate added are reported as wt/vol. Consistent with Bunesova et al. (14) supplementing free fucose with a trace amount of glucose (0.1–0.2%) resulted in higher final biomass (**Figures 1A,B**). *B. infantis* ATCC 15697 growth on free fucose as a sole carbohydrate source was reproducible and consistent across biological replicates. Co-fermentation of 0.1% glucose and 1.9% fucose resulted in significantly greater growth (final $OD_{600} = 0.38 \pm 0.09$) relative to 0.1% glucose alone (final $OD_{600} = 0.22 \pm 0.08$, $p = 9.9E\text{-}5$)

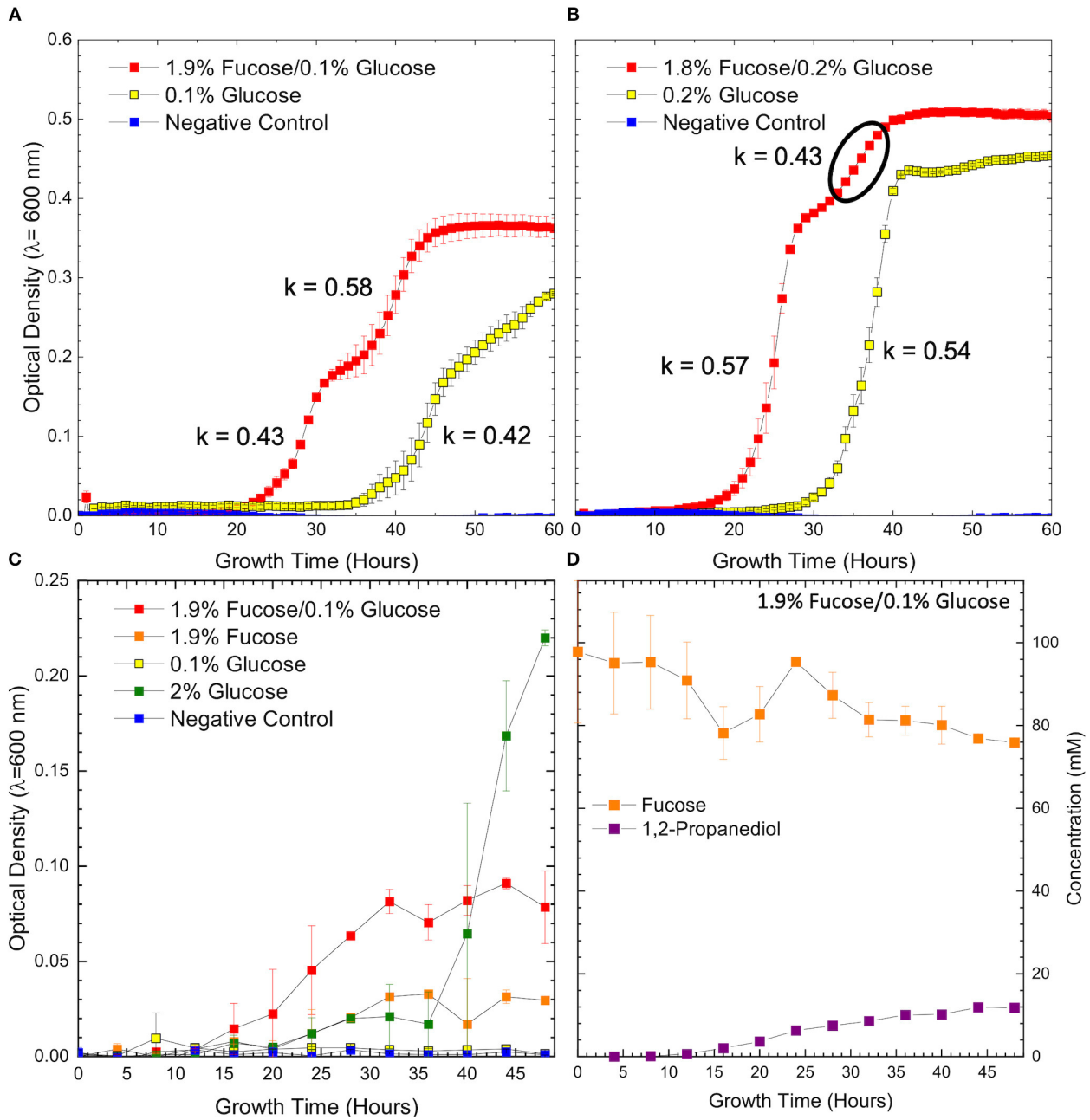

FIGURE 1 | Growth curves of ATCC 15697 in **(A)** co-fermentation of 1.9% fucose and 0.1% glucose, **(B)** co-fermentation of 1.8% fucose and 0.2% glucose and on a corresponding concentration of glucose as a sole carbohydrate source shows distinct changes in growth rate during co-fermentation. The region where growth kinetics deviate and are best fit with a second growth rate is noted by the bold circle. Growth curves of ATCC 15697 on **(C)** co-fermentation of 1.9% fucose and 0.1% glucose and on individual carbohydrate sources. Samples were analyzed for metabolite concentrations at each data point. Concentrations of fucose and 1,2-propanediol **(D)** during co-fermentation of 1.9% fucose and 0.1% glucose are inversely related and in stoichiometric agreement with the proposed fucose metabolic pathway. Error bars denote standard error of the mean.

(**Figure 1A**). Moreover, growth on 1.8% fucose and 0.2% glucose (final OD_{600} = 0.50 ± 0.06) is also significantly higher than growth on 0.2% glucose alone (final OD_{600} = 0.44 ± 0.002, p = 0.02) (**Figure 1B**). Growth on 1.8% fucose and 0.2% glucose is also significantly higher than growth on 1.9% fucose and 0.1% glucose (p = 0.003). Growth on 0.2% glucose is significantly higher than growth on 0.1% glucose (p = 2.04E-8).

Growth in co-fermentation at both concentrations of fucose and glucose exhibits two distinct regions with different growth rates. In both conditions, the growth rate in the first regime

($k = 0.43$, $k = 0.57$) is in concordance with the growth rate observed in fermentation of glucose as a sole carbohydrate source ($k = 0.42$, $k = 0.54$). Given the shift in growth rate and the relative length of each region, it is possible that the cells are transitioning from glucose to fucose metabolism as the limiting concentration of glucose is exhausted. Depletion of the preferred carbohydrate source is the most common driver of diauxic shifts in microbial systems. Interestingly, the growth rate increases across this diauxic transition during co-fermentation of 1.9% fucose and 0.1% glucose ($k = 0.43$–0.58) and decreases during co-fermentation of 1.8% fucose and 0.2% glucose ($k = 0.57$–0.43). Thus, it is possible that the initial concentration of glucose influences the direction of growth rate change. Biomass accumulation on 0.1% glucose is severely restricted indicating that *B. infantis* ATCC 15697 does not have sufficient substrate at this concentration. Therefore, it is concluded that increased growth in co-fermentation is due to the inclusion of fucose as a fermentable carbohydrate.

In order to assess metabolite production as a function of growth, larger volume co-fermentation was performed to identify intracellular metabolites from sufficient biomass. Higher volume co-fermentation of glucose and fucose (**Figure 1C**) was consistent with the smaller volume 96-well format (**Figures 1A,B**), although exhibited lower OD_{600} values overall. Again, growth in co-fermentation of 0.1% glucose and 1.9% fucose was significantly increased relative to 0.1% glucose ($p = 0.004$) and 1.9% fucose ($p = 0.02$) as a sole carbohydrate source. In the large volume format, *B. infantis* ATCC 15697 does not significantly utilize free fucose as a sole carbohydrate source relative to the negative control (i.e., no added sugar) ($p = 0.1$) (**Figure 1C**). These results are consistent with previous observations that several *B. infantis* strains do not grow efficiently on fucose and only grow in the presence of trace glucose (14).

In contrast, co-fermentation of 0.1% lactose and 1.9% fucose is driven predominately by lactose metabolism as suggested by insignificant difference to final OD_{600} relative to growth on 0.1% lactose alone (**Supplementary Figure 5**, $p = 0.07$). This is not surprising given the efficient metabolism of lactose by *B. infantis* ATCC 15697 which likely prioritizes lactose over fucose. The presence of a second growth phase in co-fermentation of lactose and fucose (**Supplementary Figure 5**) suggests that fucose is being metabolized, similar to co-fermentation of the same concentrations of glucose and fucose (**Figure 1A**). Fucose metabolism during co-fermentation with lactose is supported by 1,2-PD production and disappearance of fucose from the medium (**Figure 1D**, **Supplementary Table 1**).

1,2-Propanediol Is Produced in Stoichiometric Accordance With Free Fucose Utilization

Given that *B. infantis* ATCC 15697 co-ferments limiting concentrations of glucose or lactose and excess fucose, as well as free fucose, fermentative intermediates were profiled to clarify the fucose utilization pathway. A mechanistic pathway (**Supplementary Figure 6**) was proposed by Bunesova *et al* (14) and supported by James *et al* in *Bifidobacterium kashiwanohense* and *Bifidobacterium breve* (11). This hypothetical pathway reflects rhamnose metabolism in *Campylobacter* sp. and *X. campestris* and features several intermediate metabolites to be potentially assayed (11, 14). L-fucono-1,4-lactone was selected as a metabolite marker to track due to its stability, exclusivity to the putative fucose pathway, and it yields a single spectral peak. Conversely, L-fuconate exhibits several peaks that are interspersed among the linear form of fucose. As such, intracellular metabolites were measured during co-fermentation of 0.2% glucose and 1.8% fucose. The higher concentration of glucose was used in order to achieve sufficient biomass to reliably detect L-fucono-1,4-lactone that is expected to be present in low concentrations. The presence of a lactate, pyruvate, and 1,2-PD signal confirmed that sufficient biomass was generated to detect intracellular metabolites (data not shown).

Despite the predicted stability of L-fucono-1,4-lactone, the metabolite was not detected at any point during growth on 0.2% glucose and 1.8% fucose (**Supplementary Figure 7**). There is, however, evidence of intracellular 1,2-PD as it was observed in the GC-MS chromatogram as a shallow, broad peak appearing very close to the solvent (data not shown). If fucose is metabolized through a L-fucono-1,4-lactone intermediate it may be highly transient or present in concentrations below the limit of detection. Although intracellular L-fucono-1,4-lactone was not detected, the presence of intracellular 1,2-PD indicates that fucose metabolism proceeds during biomass accumulation. Given that 1,2-PD is a secreted metabolic end product and expected to be found in higher concentrations in the extracellular matrix relative to intracellular levels, extracellular 1,2-PD served as a marker for fucose metabolism. Accordingly, we hypothesized that *B. infantis* ATCC 15697 secretes extracellular 1,2-PD corresponding with a decrease in fucose as a hallmark of fucose metabolism (**Figure 1D**).

Unlike glucose or galactose, fucose is not predicted to enter the bifidobacterial central metabolic pathway, termed the bifid shunt. This assumption is based on fucose metabolism in other bacterial systems and on the unique characteristics of the bifid shunt. Instead, it is hypothesized that an equimolar ratio of pyruvate and 1,2-PD is produced per mole of fucose consumed. Thus, 1,2-PD would be produced at the same rate that fucose is expended. Pyruvate would be catabolized and secreted as lactate, formate, and/or acetate to recycle cofactors. Extracellular metabolites produced during growth on fucose are provided in **Table 1**. 1,2-PD is produced at 0.5 times fucose consumption during co-fermentation of fucose and glucose. During growth on fucose as a sole carbohydrate source, 1,2-PD is produced at 0.44 times the consumption of fucose. While these ratios are not the expected equimolar production, it is important to note that the uncertainty in fucose concentration is large. Accounting for uncertainty in fucose concentration provides a range of the ratio of 1,2-PD production to fucose disappearance of 0.3–1.4 during co-fermentation of fucose and glucose and 0.3–0.7 during growth on free fucose. Furthermore, some deviation from the theoretical ratio of 1 is anticipated due to variation in growth state. Overall, *B. infantis* ATCC 15697 is observed to produce 1,2-PD as a result of fucose metabolism.

TABLE 1 | *B. infantis* extracellular metabolite production during growth in co-fermentation of fucose and glucose and on fucose and glucose as sole carbohydrate sources.

	Glucose (mM)	Fucose (mM)	Lactate (mM)	Formate (mM)	Acetate (mM)	1,2-PD (mM)	Ace:Lac	Form:Ace
1.9% fucose/0.1% glucose	−5 ± 1	−26 ± 16	+1.6 ± 0.6	+11.8 ± 0.6	+15.0 ± 0.1	+13 ± 1	3.08	0.70
1.9% fucose	ND	−20 ± 7	+0.3 ± 0.8	+6.7 ± 0.9	+8.0 ± 0.7	+8.8 ± 0.2	2.26	1.02
0.1% glucose	−0.96 ± 0.09	ND	+0.10 ± 0.07	+0.1 ± 0.1	+0.58 ± 0.1	ND	0.71	0.99

Change in concentration is calculated using extracellular concentrations prior to growth and following stationary phase. ND, not detected.

TABLE 2 | *B. infantis* extracellular metabolite production during growth on 2′fucosyllactose is similar to that both in co-fermentation of fucose and lactose and on fucose and lactose as sole carbohydrates.

Carbohydrate	Lactate (mM)		Acetate (mM)		Formate (mM)		1,2-PD (mM)		Δ[Fucose] (mM)	Ace:Lac	Form:Ace
	Raw	Norm	Raw	Norm	Raw	Norm	Raw	Norm			
0.8% 2′FL	16 ± 1	16 ± 1	40.0 ± 0.8	40.0 ± 0.8	11.9 ± 0.5	11.9 ± 0.5	7.6 ± 0.1	7.6 ± 0.1	−7.5 ± 0.4	2.5	0.3
0.8% fucose	3.14 ± 0.08	18.97 ± 0.08	3.5 ± 0.2	21.3 ± 0.2	2.6 ± 0.5	15.6 ± 0.5	5.30 ± 0.08	21.01 ± 0.08	−5.3 ± 0.9	1.1	0.7
0.8% lactose	30.2 ± 0.5	22.9 ± 0.5	56 ± 1	42 ± 1	3.6 ± 0.2	2.7 ± 0.2	ND	ND	ND	1.8	0.06
0.8% fucose/0.8% lactose	28.1 ± 0.3	22.4 ± 0.3	45.5 ± 0.7	36.3 ± 0.7	3.9 ± 0.3	3.1 ± 0.3	4.07 ± 0.05	3.25 ± 0.05	−4.8 ± 0.4	1.6	0.09

Change in concentration is calculated using extracellular concentrations prior to growth and following stationary phase. ND, not detected.

The acetate:lactate ratio during co-fermentation of glucose and fucose surpasses the theoretical ratio of 1.5 secreted during hexose metabolism (**Table 1**). As compared to efficiently fermented carbohydrates such as lactose (**Table 2**), lactate concentrations greatly decreased and formate concentration increased during growth on fucose. This metabolic shift to favor formate secretion may be to prioritize ATP production through a branch in the bifid shunt. Similar observations have been reported for *B. infantis* ATCC 15697 (39) and other *Bifidobacterium* species (40) during inefficient metabolism of neutral HMOs. An acetate:lactate ratio of 0.99 during growth on 0.1% glucose may be due to poor growth on such a limiting sugar concentration, and thus deviates from the theoretical ratio of 1.5.

Increased formate:acetate ratios are consistent with a shift in pyruvate metabolism. When more than 50% acetyl-CoA is converted to acetate by *B. longum*, the theoretical formate:acetate ratio is approximately 0.4 (41). Here, *B. infantis* ATCC 15697 has surpassed that threshold, suggesting that there is a shift to produce more acetyl-CoA than lactate from pyruvate. The increased ratios are expected with the inefficient fermentation of fucose as a sole carbohydrate source. These results support the hypothesis that *B. infantis* ATCC 15697 utilizes free fucose as a carbohydrate to support growth, although fucose fermentation is somewhat inefficient under these experimental conditions.

1,2-Propanediol Is Produced in Stoichiometric Accordance With 2′FL Utilization

Similar to fucose metabolism, 2′FL is metabolized by *B. infantis* ATCC 15697 to produce 1,2-PD (**Figures 2A–C**) when fermenting substrates at slightly higher concentrations than those found in human milk (42). Each molecule of 2′FL is composed of a lactosyl and fucosyl moiety, thus utilization of 2′FL approximates fucose utilization. As with co-fermentation of fucose and glucose, 1,2-PD production would be expected to be equivalent to fucose. In three tested conditions (0.8% 2′FL, 0.8% fucose, and 0.8% fucose/0.8% lactose) the decrease in fucose or 2′FL concentration is nearly identical to the production of 1,2-PD (**Table 2**). In both instances formate production is increased fivefold (5.8-fold for free fucose and 4.4-fold for 2′FL) in a comparison of free fucose and 2′FL metabolism (**Table 2**). Moreover, lactate production is decreased relative to growth on lactose when normalized to final OD_{600}. In contrast, acetate production is reduced while subsisting on fucose which could be an artifact of poor growth or otherwise confounded due to metabolism of the lactosyl component of 2′FL. A co-fermentation of 0.8% fucose and 0.8% lactose was used to functionally approximate fermentation of integrated 0.8% 2′FL as a sole carbohydrate source (**Table 2**). Whereas, absolute metabolite concentrations of the fucose/lactose co-fermentation are in closer agreement with those during growth on 2′FL, the formate and 1,2-PD concentrations during fucose metabolism are reduced with the addition of the more efficiently utilized lactose. As such, it is difficult to derive conclusions regarding free fucose metabolism in direct comparison to 2′FL-derived fucose.

Limiting Concentrations of Mono- and Disaccharide Residues of 2′FL Increase Fucose Metabolism

In order to clarify the boundaries of fucose metabolism in the presence of a preferred sugar, *B. infantis* was grown in co-fermentation of excess fucose and limiting concentrations of lactose, glucose, and galactose. Initial co-fermentation of limiting lactose (0.1%) (**Supplementary Figure 5**) resulted in variable growth indicating that this concentration is too low to consistently support growth. Thus, co-fermentation of 2% fucose

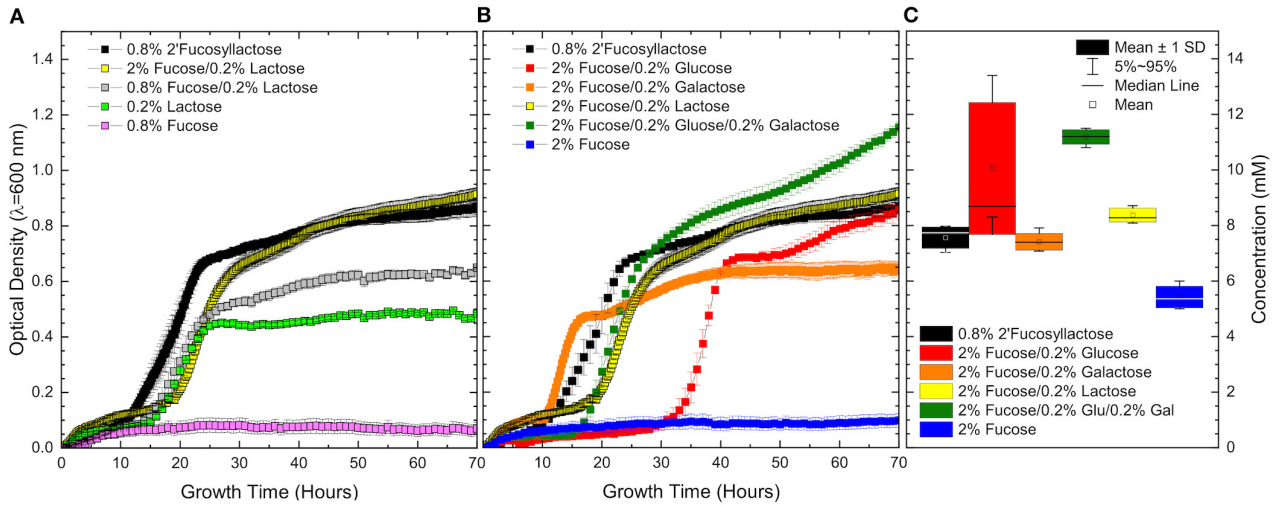

FIGURE 2 | Growth curves of ATCC 15697 growth in **(A)** co-fermentation of excess fucose and limiting lactose as compared to 2′fucosyllactose. Co-fermentation of fucose and limiting lactose shows similar behavior to fermentation of 2′fucosyllactose and results in increased growth over individual carbohydrate sources. Co-fermentation of **(B)** excess fucose and limiting concentrations of mono- and di-saccharide components of 2′fucosyllactose shows a consistent synergistic effect. 1,2-Propanediol production **(C)** during co-fermentation is equal to or higher than 2′fucosyllactose and higher than growth on fucose as a sole carbohydrate source. Error bars denote standard error of the mean.

and slightly higher concentrations (0.2%) of glucose, galactose, and lactose were used (**Figure 2B**). Co-fermentation of fucose, glucose, and galactose produced the highest biomass as measured by final OD_{600} (**Figure 2B**). Co-fermentation of fucose with glucose or lactose produced roughly equivalent final OD_{600} as fermentation of 2′FL as the sole carbon source. In contrast, co-fermentation of fucose and galactose produced a lower final OD_{600}. Regardless of the co-fermented substrate, growth in co-fermentation with fucose achieved higher biomass than growth on fucose or the limiting carbohydrate alone. Furthermore, the final OD_{600} achieved in co-fermentation was higher than the sum of final OD_{600} achieved during growth on individual components as sole carbohydrate sources (lactose shown in **Figure 2A**, additional sugars not shown).

In accordance with increased growth, final 1,2-PD concentrations increased during growth in co-fermentation relative to growth on 2% fucose as a sole carbohydrate source (**Figure 2C**). All co-fermentations exhibit significantly higher 1,2-PD secretion relative to growth on fucose alone [fucose/glucose (p = 6.76E-8), fucose/galactose (p = 0.02), fucose/lactose (p = 1.24E-4), fucose/glucose/galactose (p = 1.79E-8)]. Moreover, 1,2-PD production was unchanged (galactose or lactose) or increased [glucose (p = 0.003), glucose/galactose (p = 1.65E-7)] during co-fermentation as compared to 1,2-PD production during growth on 0.8% 2′FL. Fucose metabolism and central carbohydrate metabolism are linked as evident from increased final OD_{600} corresponding to increased 1,2-PD concentration.

Clearly fucose is not a preferred carbohydrate source for *B. infantis* ATCC 15697 and is not efficiently metabolized as a sole carbohydrate source regardless of the addition of limiting co-fermented sugars. Despite this inefficient metabolism, co-fermentation significantly increases growth and hallmarks of fucose metabolism. This phenomenon was also observed by Bunesova *et al* in that bifidobacterial growth on free fucose as a sole carbohydrate source was greatly improved by the inclusion of trace (0.4 mM) glucose (14). It is possible that a metabolite represses the fucose pathway as a result of fucose catabolism or carbon flux through the bifid shunt. James et al. putatively linked a LacI-family regulator in *B. kashowanihense* (*fumR)* to fucose metabolism, although mechanistic details of regulatory interactions remain unresolved (11). *B. infantis* ATCC 15697 possesses a homolog of *fumR* (Blon_RS01775) and this putative repressor may restrict fucose metabolism in the presence of sufficient concentrations of efficiently utilized sugars (e.g. glucose, galactose, and lactose). It is possible that fucose transport is repressed in the presence of high concentrations of efficient substrates, similar to the preferential metabolism of lactose over glucose observed in *B. longum* (43). In this experimental system, fucose transport is likely not hindered by excess lactose, as extracellular fucose concentrations diminish uniformly regardless of growth solely on fucose or in co-fermentation with lactose (**Table 2**). The variability within LacI-family allosteric activities suggest that bifid shunt and/or fucose-derived metabolites potentially regulate fucose metabolism (44). Furthermore, fucose metabolism is not repressed by limiting co-fermented carbohydrates. This is evidenced by similar concentrations of secreted 1,2-PD on excess 2′FL and excess fucose co-fermented with limiting sugars.

Utilization of Higher Concentrations of 2′FL Does Not Promote Efficient *B. infantis* Growth

2′FL concentrations in human milk vary between and within populations depending on multiple factors including maternal

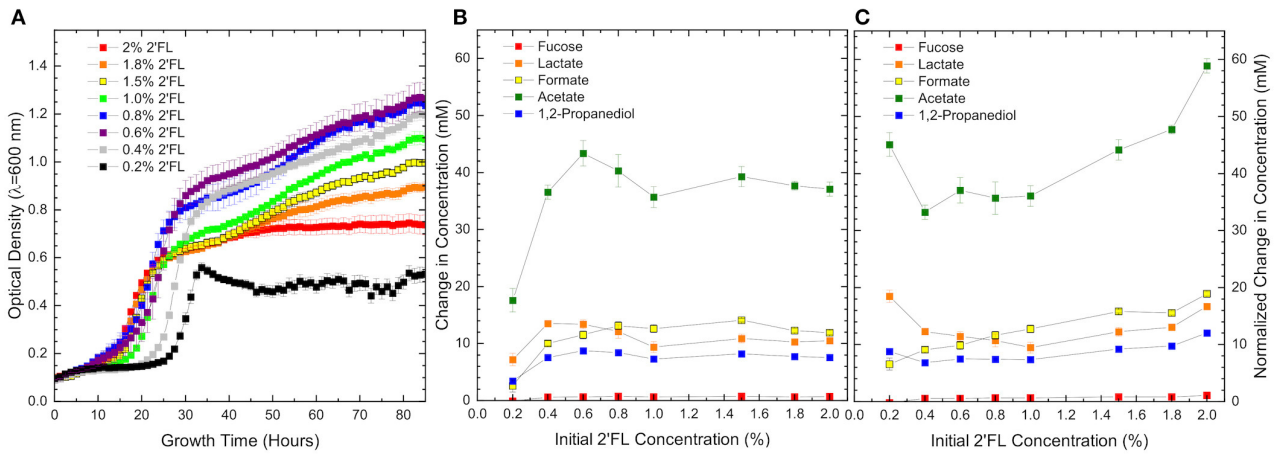

FIGURE 3 | Growth curves of ATCC 15697 growth on **(A)** 2′fucosyllactose as a sole carbohydrate source is highly concentration-dependent. Metabolite production **(B)** appears to be consistently dependent on 2′fucosyllactose concentration, but concentration dependence does not persist when **(C)** metabolite concentrations are normalized to account for differences in biomass. Error bars denote standard error of the mean.

TABLE 3 | *B. infantis* 2′fucosyllactose consumption and 1,2-propandiol production during growth on 2′fucosyllactose as a sole carbohydrate source is not concentration-dependent when differences in final biomass are accounted for. However, acetate:lactate and formate:acetate ratios show some subtle variation with 2′fucosyllactose concentration.

2′FL conc. (%w/v)	2%	1.8%	1.5%	1%	0.8%	0.6%	0.4%	0.2%
Final OD_{600} (± stdev	0.63 ± 0.04	0.79 ± 0.02	0.89 ± 0.01	0.99 ± 0.02	1.13 ± 0.01	1.17 ± 0.06	1.10 ± 0.02	0.39 ± 0.03
Acetate:Lactate	3.2	3.2	3.3	3.3	2.9	2.9	2.4	2.0
Formate:Acetate	0.34	0.34	0.37	0.37	0.34	0.29	0.31	0.24
Δ[2′FL] (mM)	−7 ± 2	−5 ± 2	−2 ± 1	−6 ± 2	−7 ± 1	−8 ± 2	−11.1 ± 0.3	−7 ± 2
Δ[1,2–PD] (mM)	+7.57 ± 0.07	+7.7 ± 0.1	+8.2 ± 0.2	+7.29 ± 0.05	+8.4 ± 0.4	+8.7 ± 0.4	+7.5 ± 0.1	+3.4 ± 0.2
Normalized Δ[2′FL] (mM)	−14 ± 2	−7 ± 2	−2 ± 1	−6 ± 2	−6 ± 1	−7 ± 2	−10.1 ± 0.3	−17 ± 2
Normalized Δ[1,2-PD] (mM)	+12.02 ± 0.07	+9.8 ± 0.1	+9.2 ± 0.2	+7.36 ± 0.05	+7.4 ± 0.4	+7.5 ± 0.4	+6.8 ± 0.1	+8.8 ± 0.2

Change in extracellular concentration is calculated using concentrations prior to growth and following stationary phase. ND, not detected.

secretor status (6, 7, 42, 45–47). In order to test the hypothesis that the fucose utilization phenotype responds to 2′FL in a concentration-dependent manner, *B. infantis* ATCC 15697 was grown on 2′FL as the sole carbohydrate source at concentrations ranging from 0.2% (i.e., limiting) to 2% (i.e., exceeds human milk concentrations). Thus, concentration-dependent growth on 2′FL would result in higher final OD_{600} and higher 1,2-propanediol production. Interestingly, and counter to this hypothesis, 2% 2′FL promotes moderate *B. infantis* ATCC 15697 growth, reaching an OD_{600} of 0.64 ± 0.06. Accumulated biomass increases with diminishing 2′FL concentrations (**Figure 3A**) until 2′FL is limiting to exhibit an inverse relationship. A more pronounced diauxic curve is exhibited as 2′FL concentration falls below 1% and is suppressed again at concentrations below 0.6%. It is currently unclear how this concentration-dependence relates to fucose metabolism.

Absolute concentrations of secreted metabolites follow the parabolic trend observed in final OD_{600} (**Figure 3B**); however, this trend does not hold when metabolite concentrations are normalized to final OD_{600} to account for biomass variation (**Figure 3C**). As a result, the discordance between cellular growth and metabolite production is apparent and indicates a significant biological phenomenon. When normalized to biomass, formate is secreted in a consistently positive slope with increasing 2′FL. Acetate production also exhibits a net positive slope except at the lowest 2′FL concentration, which is severely limiting. The increase in formate and acetate production suggest that metabolic flux increases along with 2′FL concentrations. Formate production is small relative to the increase in acetate concentrations coinciding with increasing 2′FL concentration and, thus, does not significantly change the formate:acetate ratio (**Table 3**). Whereas normalized formate and acetate production are concentration-dependent, normalized lactate production does not exbibit a consistent relationship with 2′FL concentration. The relatively constant lactate concentration is consistent with increased formate and acetate production given that these metabolites are often increased at the expense of lactate production (41, 48–51). The specific metabolic end products that were quantified were not significantly impacted by 2′FL concentration despite significant changes in biomass accumulation with changing 2′FL concentration.

A potential factor in inhibition of growth with increasing 2′FL concentration is a potential toxicity of 1,2-PD, as 1,2-PD

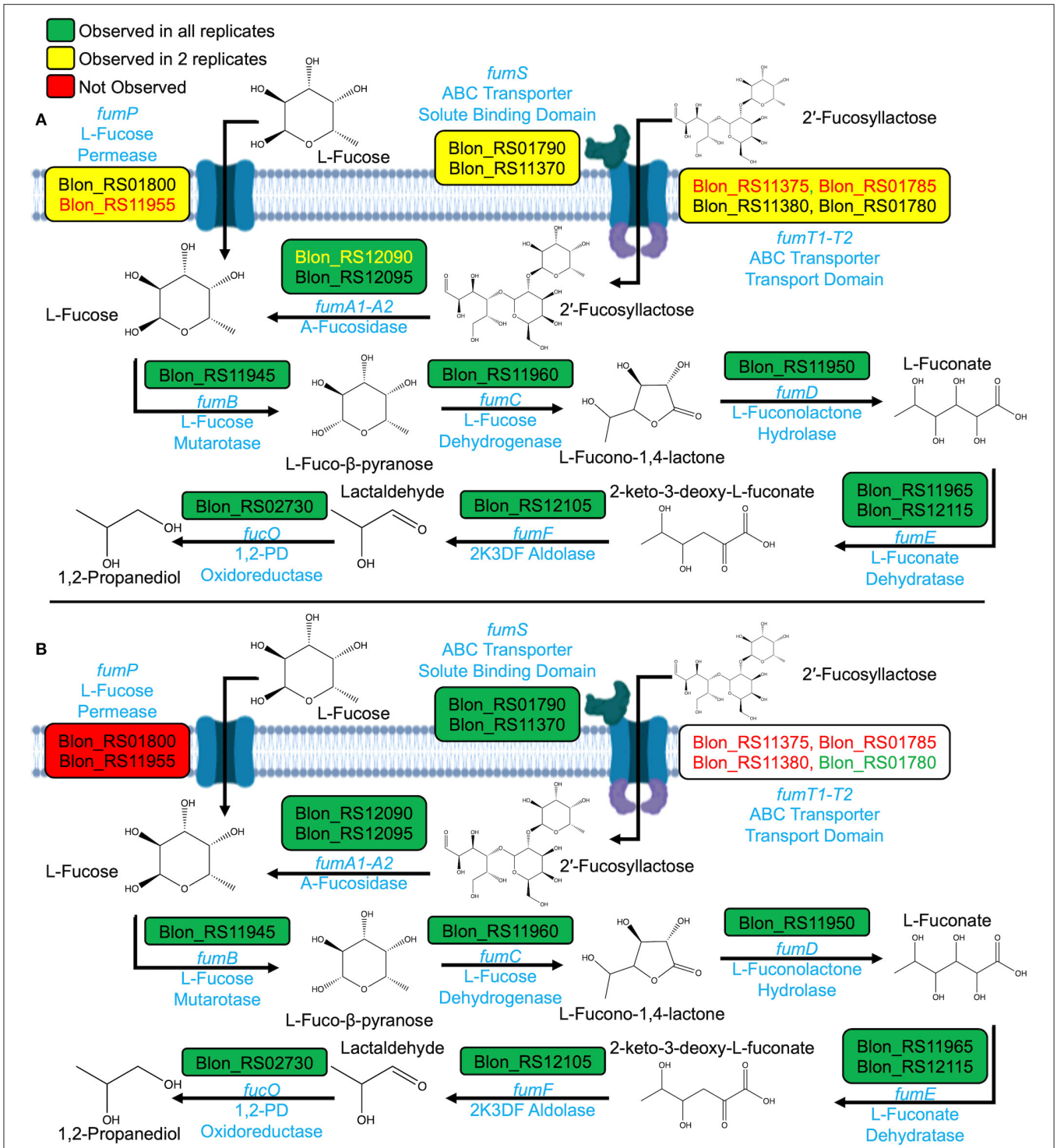

FIGURE 4 | Proposed fucose pathways based on proteome during growth on **(A)** free fucose and **(B)** 2% 2′fucosyllactose as sole carbohydrate sources. Gene symbols are given in light blue above the reaction arrows and corresponding protein names below.

concentration increases with that of 2′FL (**Figure 3B**). To test this, a growth inhibition assay indicated that 1,2-PD is only slightly inhibitory between 168 and 0.08 mM with a difference in OD_{600} of 0.107 ± 0.007 (p = 9.5E-8) between the highest and lowest 1,2-PD concentrations. Similarly, the growth inhibition assay of lactate (i.e., inhibitory at high concentrations)

indicates a similar slight inhibition between 144 and 0.07 mM lactate (ΔOD_{600} of 0.150 ± 0.005, p = 3.0E-7). Neither metabolite accumulates to inhibitory concentrations prior or during growth. 1,2-PD reaches a maximum concentration of 8.6 ± 0.4 mM at 0.6% 2′FL. Therefore, it is unlikely that secreted 1,2-PD or other quantified end-product metabolites impact growth at higher concentrations of 2′FL. It is possible that fucose metabolism is repressed by some unknown mechanism. Thus, proteomics and transcriptomics were conducted to examine global regulatory networks during fucose metabolism to better understand the relationship between fucose, lactose, and 2′FL utilization.

The Proteome Contains Hallmarks of Putative Bifidobacterial Fucose Metabolic Pathway

The *B. infantis* proteome was interrogated for signals of pathways active during fucose catabolism. The proteome during growth on free fucose as a sole carbohydrate exhibits features consistent with the pathway proposed for *B. kashowanohense*, albeit yet to be validated incisively (**Figure 4A**) (11, 14) **Figure 4** integrates proteins within the proteome encoded by the genes of interest in the hypothetical *B. infantis* fucose metabolic pathway. Putative fucose metabolism proteins were observed in at least two of three biological replicates. Transport-associated proteins were identified in relatively low abundance, which may be due to their spanning the cell membrane and, thus, tendency to collapse in the insoluble proteome fraction. Nevertheless, transport proteins were observed in two of three biological replicates.

An ABC transporter linked to 2′FL (Blon_RS01780-RS01790) (4) is expressed during growth on fucose as a sole carbohydrate. This indicates that fucose signals 2′FL metabolism to initiate and reflects likely overlapping processes to catabolize free or integrated fucose. Partial evidence of a secondary transporter (Blon_RS11370-RS11380) was identified in the proteome. Proteins corresponding to Blon_RS11370 (FumS) and Blon_RS11380 (FumT2) were detected in two of three replicates, and Blon_RS11375 (FumT1) was not observed.

The fucose proteome is in agreement with 1,2-PD production (**Table 1**). Although intracellular L-fucono-1,4-lactone was not detected *via* GC-MS, proteins with KEGG orthologs corresponding to L-fucono-1,4-lactone and L-fuconate synthesis were present in the proteome. These proteins with putative fucose metabolic functions include L-fucose dehydrogenase (FumC), L-fuconolactone hydrolase (FumD), as well as L-fuconate dehydratase (FumE). Furthermore, differential expression of L-fuconolactone and L-fuconate-related proteins was observed during growth on free fucose as compared to growth on lactose. FumD (Blon_RS11950) showed a $\log_2$ fold change of 13.1 (p = 5.95E-7) and two orthologs of FumE (Blon_RS11965 and Blon_RS12115) showed $\log_2$ fold changes of 15.4 (p = 1.39E-5) and 1.7 (p = 0.02). If these enzymes function as predicted, L-fucono-1,4-lactone and L-fuconate may be produced as highly transient intermediates during fucose metabolism.

2′FL Induces a Proteomic Signature That Reflects a Shared Metabolic Pathway With Free Fucose

The comparison between the proteomes of two 2′FL concentrations of free fucose indicate close similarity between growth conditions (**Figure 4B**). Differential expression of FumD (Blon_RS11950) ($\log_2$ fold change 12.1, p = 0.004) and FumE (Blon_RS11965) ($\log_2$ fold change 14.0, p = 9.75E-6) are also observed during growth on 2% 2′FL as compared to lactose. Unlike the free fucose proteome, L-fucose permease (FucP) was not detected during growth on 2′FL. Thus, it is likely that there is a single metabolic pathway for fucose catabolism once fucose is translocated across the cell envelope by *B. infantis* ATCC 15697. This is based on expression of the same fucose-associated proteins regardless of conditions and variation in the specific transport proteins detected. During growth on 2% 2′FL, two orthologs of FumS (Blon_RS01790 and Blon_RS11370) show significantly increased expression relative to lactose ($\log_2$ fold change 12.6, p = 0.0004; 9.3, p = 0.005, respectively). FumS was not seen to be significantly differentially expressed during growth on fucose as compared to lactose. Interestingly, fucose may induce the cell to translocate 2′FL *via* dedicated ABC transporters, whereas 2′FL may not provide the converse signal to transport fucose through FucP.

Varying 2′FL Concentration Does Not Significantly Alter the *B. infantis* Proteome

2′FL concentration impacts the *B. infantis* growth phenotype (**Figure 3A**) which is not fully reflected in normalized metabolite concentrations (**Figures 3B,C**). Using the proposed pathway in *B. kashowanohense* as a reference (11), two 2′FL concentrations yielded highly similar proteomes at 2% and 0.8% 2′FL as depicted in **Figure 4B** and **Supplementary Figure 8**, respectively. FumT2 provides a minor exception in that the 2′FL-associated transport permease was identified with high confidence in all 2% 2′FL replicates, and 2 of 3 at 0.8% 2′FL. It is likely that this is not biological variation, rather due to low abundance of the protein. Fucose metabolism-related proteins are not significantly differentially expressed during growth on 0.8% 2′FL as compared to growth on 2% 2′FL during mid-log phase. It cannot be excluded that varying 2′FL concentrations impact relative levels of expressed protein at later stages in growth, when the divergence of growth phenotypes is more pronounced.

2′FL Induces Fucose-Associated Gene Expression in the *B. infantis* Transcriptome

Regardless of 2′FL concentration (0.8 and 2%) the *B. infantis* ATCC 15697 transcriptomes are highly similar, as observed for their cognate proteomes. This is evidenced by low sample-to-sample distance between the two 2′FL concentrations and over two-fold higher sample-to-sample distance between 2′FL and lactose fermentations (**Figure 5A**) as well as clustering in the PCA plot (**Supplementary Figure 9**). Volcano plots confirm this observation as well (**Figures 5B,C**). There are relatively few genes differentially expressed between 2′FL concentrations (**Figure 5D**). Despite efforts to scale-up, the inefficient growth

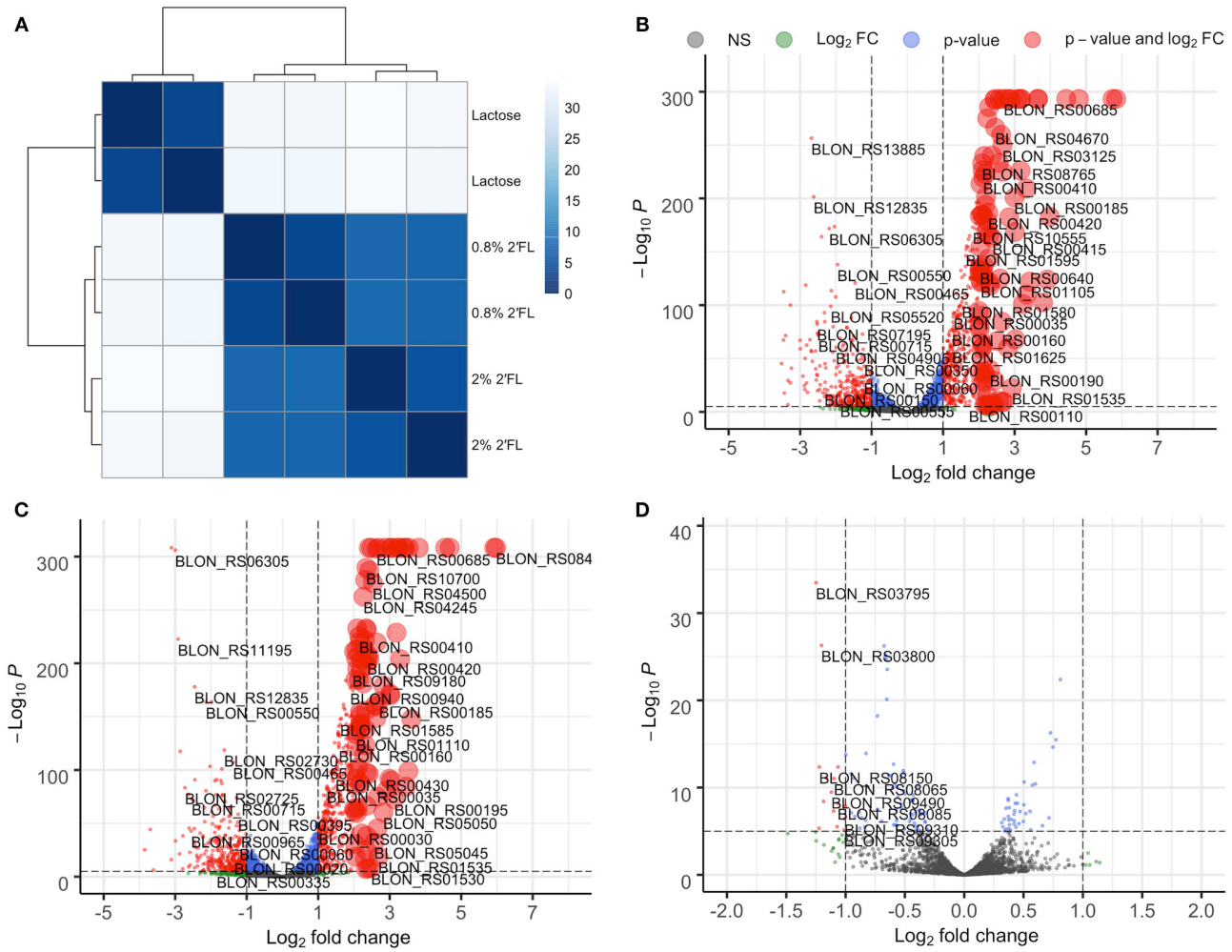

FIGURE 5 | *B. infantis* gene expression is similar during growth on 0.8% and 2% 2′fucosyllactose as compared to during growth on lactose as seen from **(A)** sample-to-sample distance. While gene regulation is similar, there are several genes that show concentration-dependent regulation when volcano plots are used to compare **(B)** 0.8% 2′fucosyllactose and lactose, **(C)** 2% 2′fucosyllactose and lactose, and **(D)** 0.8% and 2% 2′fucosyllactose.

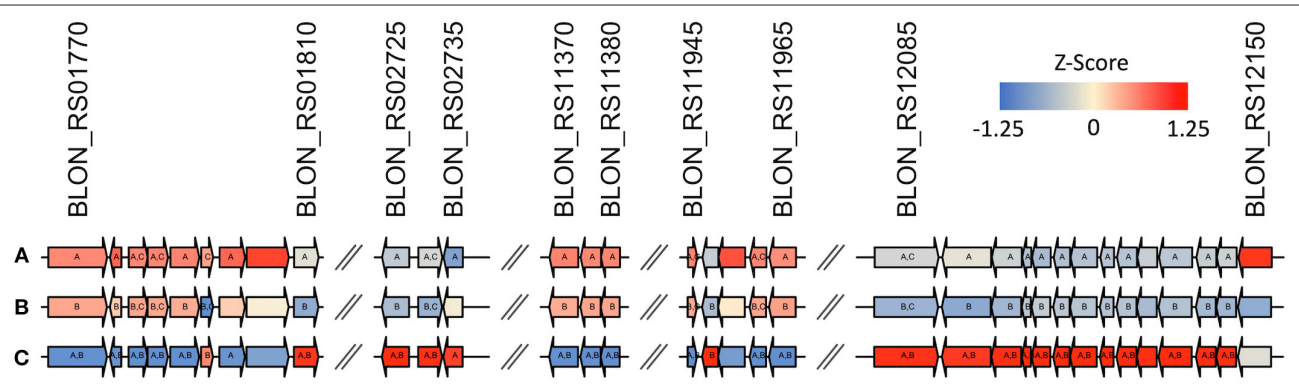

FIGURE 6 | Gene maps show that there is differential regulation across the entire fucose metabolic pathway during growth on **(A)** 0.8% 2′fucosyllactose, **(B)** 2% 2′fucosyllactose, and **(C)** lactose as described by z-scores calculated from normalized count data. Gene annotations and predicted functions are provided in **Supplementary Table 2**.

on free fucose did not generate sufficient quality and quantity of RNA for transcriptomics.

Interestingly, 2′FL induces differential expression of the majority of proposed fucose metabolism genes relative to lactose ($p \leq 0.01$; **Figure 6**, **Supplementary Figures 10**, **11**). The exceptions are fuconate dehydratase (Blon_RS01795), fucose permease (Blon_RS11955), and solute-binding protein *fumS* (Blon_RS12150) which did not exhibit significant differences. These genes may not be expressed, or they may have similar constitutive expression levels between conditions. In addition, these three genes have multiple copies within the ATCC 15697 genome. Additional *fumE* paralogs are differentially expressed, with Blon_RS11965 highly upregulated ($\log_2$ fold change >4) whereas Blon_RS12115 is downregulated ($\log_2$ fold change<-1.5). Blon_RS01810 encodes another putative fucose permease which is downregulated ($\log_2$ fold change<-0.65, $p \leq 0.001$). Blon_RS01790 ($\log_2$ fold change>3), Blon_RS11370 ($\log_2$ fold change>1.3), and Blon_RS12135 ($\log_2$ fold change < -1.4) encode *fumS*-like genes and are differentially regulated. There are several potential explanations for these observations, and they likely all contribute uncertainty. Foremost is that the putative *B. kashowanohense* pathway is incompletely characterized and validated including the constituent genes and their activities. Moreover, *B. infantis* fucose metabolism may diverge from *B. kashowanohense*, and paralogs may have multiple functions potentially unrelated to fucose metabolism.

Despite differential regulation between 2′FL relative to lactose, the transcriptomes remain relatively static regardless of 2′FL concentration (**Figure 6**). Two putative *fumS* genes (Blon_RS01790, $p = 0.004$ and Blon_RS12150, $p = 0.002$) and a *fumT1* gene (Blon_RS01785, $p = 0.01$) are down-regulated on 2% 2′FL relative to 0.8% 2′FL (**Figure 6**). This down-regulation may reflect the lower growth observed on higher concentrations of 2′FL. In addition, 2% 2′FL down-regulates a fucose dehydrogenase (Blon_RS11960; $p < 0.01$) and fuconate dehydratase (Blon_RS11965; $p < 0.05$). This down-regulation is consistent with lower growth on 2′FL. Similarly, a putative lactaldehyde reductase (Blon_RS02730; $p < 0.0001$) was downregulated during growth on 2% relative to 0.8% 2′FL. This enzyme could catalyze the conversion of lactaldehyde to 1,2-PD. Thus, differential regulation of fucose-related genes may reflect the non-linear relationship between 2′FL concentration and growth phenotype.

Differential regulation is not limited to the fucose-related genes previously identified. Given that the changes in metabolite production are not specific to fucose utilization (i.e., acetate, lactate, and formate) between 2′FL and lactose (**Table 2**), differential expression in the central pathway was examined. The majority of genes involved in the central pathway are up-regulated during growth on 2′FL as compared to lactose (**Supplementary Figure 12**). ROK family glucokinase Blon_RS02865 ($p < 0.0001$) and acetaldehyde-CoA/alcohol dehydrogenase Blon_RS11570 ($p < 0.001$) are both down-regulated during growth on 2′FL as compared to growth on lactose at both concentrations of 2′FL. Reduced glucokinase and acetaldehyde-CoA/alcohol dehydrogenase expression was also observed in *B. kashiwanohense* APCKJ1 during growth on 2′FL as compared to growth on both sorbitol and lactose (11). Genes implicated in sialic acid metabolism are similarly down-regulated in a consistent manner between both concentrations of 2′FL as compared to lactose (**Supplementary Figure 13**). This suggests that metabolism of the neutral fucosylated 2'FL regulates acidic HMO metabolism. It is unclear if this is a feature of all fucosylated HMOs or unique to the abundant 2'FL oligosaccharide species.

CONCLUSIONS

B. infantis ATCC 15697 metabolizes both free and HMO-derived fucose through a common and likely phosphorylation-independent pathway to produce 1,2-PD. During fucose metabolism, carbon is shunted from lactate and toward formate and 1,2-PD production. This metabolic shift differs somewhat during free fucose metabolism relative to that of 2′FL. Although the inefficient metabolism of free fucose hinders direct comparisons in this respect. The shift to prioritize formate and 1,2-PD production may impact trophic relationships within the infant gut microbiome. Accordingly, it has been reported that the infant-associated *Eubacterium hallii* metabolizes 1,2-PD to produce short chain fatty acids butyrate, formate, and propionate. All of which potentially benefit the gut microbiome and overall infant development (52, 53). When presented with equal concentrations of fucose and lactose, *B. infantis* ATCC 15697 preferentially metabolizes the preferred lactose with minimal fucose utilization. Conversely, *B. infantis* ATCC 15697 exhibits significantly more growth on excess fucose with limiting concentrations of preferred carbohydrates, relative to individual carbohydrate components. It is clear that fucose catabolism is interconnected with the bifid shunt given the production of end-products typically secreted by bifidobacteria. The mechanistic relationship between the feeder fucose pathway and the bifid shunt is yet to be fully validated. Bifidobacterial metabolism of fucose may provide additional protection against enteric pathogens, which are known to utilize fucose cleaved from host mucin. Continued investigation of fucosylated HMOs and co-fermentation of free fucose in combination with other carbohydrate sources will enable greater clarity in this regard. Moreover, investigating additional *B. infantis* strains, as well as other species, will generalize observations to inform *in vitro* and *in vivo* host-microbial models in an effort to characterize metabolic networks between the infant and its microbiota. Ultimately, the goal is to understand this system with scientific rigor to potentially innovate precision nutrition interventions to support infant health and development.

DATA AVAILABILITY STATEMENT

Data are available *via* NCBI GEO under accession GSE159189 and *via* ProteomeXchange with identifier PXD021868.

AUTHOR CONTRIBUTIONS

LRD and DAS conceived, designed the study, wrote, revised, and approved the submitted manuscript. LRD, EÖ, and AR generated data. LRD, EÖ, AR, and DAS analyzed data. LRD drafted the initial manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Cow's Milk and Dairy Consumption: Is There Now Consensus for Cardiometabolic Health?

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Cow's milk and dairy products derived from this complex food source have long been proposed as beneficial to human health, yet underlying clinical evidence of direct benefit continues to raise controversy. Limited evidence supports positive cardiometabolic effects of a number of dairy macro- and micronutrient components including whey protein and casein, unsaturated fats, milk fat globule membrane (MFGM) and polar phospholipids, vitamin D and calcium, in addition to non-bovine components including bacterial and yeast probiotics. More controversial remain lipid components *trans* fats, including *trans* vaccenic acid, *trans* palmitoleic acid, and conjugated *cis trans* linoleic acid (CLA), plus medium-chain and odd-chain dairy fats. New evidence is rapidly identifying multiple pathways by which these dairy nutrients may effect health. Processing, including fermentation and homogenization, may also have positive effects. Conversely, the high saturated fat content of dairy has long raised concern, aligned with international guidelines to minimize dietary intake of animal-origin saturated fatty acids (SFA) to achieve better cardiometabolic health. However, led in part by observational studies and meta-analyses showing dairy to have no or even an inverse association with cardiometabolic health, evidence from randomized controlled trials (RCTs) has been scrutinized over the last 5 years, and focus on low-fat dairy has been challenged. Recent evidence supports the hypothesis that adverse effects of SFAs on metabolic health may be ameliorated when these fats are consumed within a complex matrix such as milk, cheese or yogurt, and that dairy food categories may influence outcomes as much as total fat content. For example, yogurt and high-fat, high-SFA cheese have a negative association with risk of type 2 diabetes (T2D) in many, not all, published trials. However, large sample dairy RCTs of long duration with CVD or T2D incidence as primary endpoints are lacking. This is a clear research gap, with these clinical studies required if a causative link between dairy and improved cardiometabolic health is to be confirmed and in turn promoted through dietary guidelines. Current advisories from national guidance groups such as American Heart Association (AHA) and European Society of Cardiology (ESC) continue to promote consumption of low-fat dairy products, whilst liquid milk and yogurt remain part of nutrition guidelines from joint American Diabetes Association (ADA)/European Association for Study of Diabetes (EASD) reports, and as part of a "no-one-size-fits-all" answer to diet and T2D by the ADA in their most recent 2019 Consensus Report.

Keywords: fat, protein, dairy, CVD, diabetes, cardiometabolic

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INTRODUCTION

Cow's milk and dairy products derived from this complex food source have long been proposed as beneficial to human health, yet underlying clinical evidence supporting direct benefit to cardiometabolic health continues to raise controversy, based primarily on the high saturated fatty acid (SFA) content of whole-fat dairy. Whether high levels of dietary SFAs *per se* do indeed initiate a cascade of worsening intermediary blood markers including an adverse lipoprotein profile, and in turn lead to higher prevalence of cardiometabolic disease is now under considerable scrutiny. This narrative review aims to present the most recent evidence from both observational cohorts and randomized controlled trials (RCTs) that investigate the relationships between dairy and risk of cardiovascular disease (CVD) and type 2 diabetes (T2D), and to evaluate the evidence provided by these different study types. Also to identify, where possible, potential mechanisms by which dairy nutrients may promote health benefits.

Concern around dairy and potential adverse health outcomes arose from early epidemiologic data which supported a strong association between animal-origin food groups that provide a major source of dietary SFAs and an increased risk of CVD (1). In countries such as the UK, milk and dairy foods contribute almost 30% of SFA intake, and so quite reasonably have been considered a food component of concern (2). However, current literature shows growing support for the proposal that dairy products may have a neutral or even positive effect on CVD outcomes (3), with a number of meta-analyses supporting this relationship (4–9). There is also a new but growing consensus that the matrix of a whole-fat food such as dairy may be more important than the content and composition of component isolated fatty acids. Such that food-based rather than nutrient-based recommendations should be developed for CV health (3, 10, 11). It is notable however that the predominance of this evidence is obtained from observational studies. There is less evidence from RCTs, particularly with reference to incident CVD where long-term dairy interventions evaluating hard CV event points are lacking. This may be critical when aiming to interpret findings suggestive of a positive relationship with regular- and high-fat diary, and in order to develop robust public health recommendations.

A number of meta-analyses and systematic reviews have also focused on dairy and T2D (4, 12–17), again showing an inverse association between dairy intake and risk of T2D in observational studies. A recent expert panel position paper (18) reported a number of key findings including the evidence from large prospective cohort studies that total dairy consumption has a neutral or moderately beneficial effect on T2D risk. Again notably this is an outcome supported only by limited evidence from randomized controlled trials (RCTs) (19), with no long-term interventions investigating the effect of high-fat dairy on incident T2D. Dairy category is clearly important, again with evidence from prospective cohort studies showing fermented yogurt to be most strongly associated with lower T2D risk. There is with sparse evidence from RCTs. Even in 2020, the balance of evidence is predominantly from observational studies. Larger and longer duration clinical intervention trials are needed, with incident T2D as the primary outcome. RCTS are also required for better understanding of the underpinning mechanisms by which dairy may potentially be protective.

Dairy composes about 10% of the energy consumed in, for example, a typical North American diet, of which approximately half is from fluid milk, half from fermented (or "cultured") cheese, and a small percentage from fermented yogurt (20). Important nutrients found in the myriad of dairy formats include milk proteins, calcium, magnesium, potassium, medium- and odd-chain saturated fats, specific fatty acids, and low-glycemic index (GI) sugars; shown to have beneficial effects on aspects of glucose control, insulin secretion, insulin sensitivity and/or T2D risk (21) as well as a range of CV risk factors (22). Notably a number of authors (23–26) have recently highlighted the importance of focusing on foods and dietary patterns rather than simply individual dietary nutrients when assessing CVD and T2D risk. In turn it is clear that expanding response to these dietary patterns beyond simple body fatness and circulating blood lipids into the multiple risk outcomes now identified as important to CV health is important.

METHODS

A literature search was conducted using MEDLINE (via PubMed) to identify observation cohorts and intervention trials that investigated the association between dairy consumption and cardiometabolic health. Medical Subjects Headings (MeSH) included dairy OR milk OR butter OR cheese OR yogurt AND CVD OR diabetes; reviews, including systematic reviews, meta-analyses, umbrella reviews, narrative reviews.

CARDIOMETABOLIC EFFECTS OF DAIRY MACRO- AND MICRO-NUTRIENTS: EVIDENCE FROM CLINICAL STUDIES

Dairy Proteins

Whey and Casein

Among the various types of animal-based protein foods, a higher intake of protein-rich dairy products has been reported to be associated with a beneficial relationship with a range of CV endpoints in addition to glucose regulation and T2D risk reduction (16, 19, 27, 28). Milk proteins can be categorized in the simplest terms into two groups, based on solubility, as serum or whey proteins and caseins. The whey proteins remain soluble at pH 4.6 and 20°C, whilst the caseins (or "curds") precipitate out. Molecular and physicochemical properties of whey protein and caseins are highly varied, with the ratio of total whey:total casein in cow's milk of ∼20:80% (29).

Although a number of large observational cohort studies do support the association between higher intake of dairy foods and neutral or lower risk of adverse CV health, data is sparse from observational studies for whey or casein *per se*. There however are several systematic reviews of RCTs that identify positive effects of whey protein. Wirunsawanya et al., recently reported on whey supplementation and CV endpoints in 9 RCTs of overweight and obese cohorts, and identified an improvement

in serum lipoproteins for total and HDL-cholesterol in addition to improvement in blood pressure (30), although notably these effects were likely driven by body weight loss. There has been a growing literature on positive effects of whey protein on hypertension, where mechanisms are purported to include angiotensin converting enzyme (ACE) inhibition, normalization of endothelial function and opioid receptor-dependent effects, although not all studies show positive long-term outcomes (31). Recently a comprehensive systematic review by Badely et al. >2,000 individuals from 37 published RCTS, again in overweight and obese adults, showed whey protein administered in multiple forms including protein isolate, concentrate, extract, supplement and hydrolysate had a positive outcome on several CV markers. They reported a decrease in fasting triglycerides and blood pressure, but also an adverse decrease in HDL-cholesterol (32). Whilst not reporting change in body weight these outcomes were accompanied by a decrease in waist circumference, which is an indirect measure of central adiposity expected to also be accompanied by weight loss. Whey protein has long been shown to have modest positive effects on body composition during weight loss (33), but whether there are effects of whey protein on CV endpoints independent of change of body weight remains to be convincingly demonstrated. Previous observational studies have reported weight independent effects of total protein intake on cardiometabolic health, with authors in turn noting that the role of protein in CV health likely depends on the specific protein source (34).

Evidence that protein-rich dairy products may be beneficial for T2D has largely come from observational studies (27) where again attribution of positive effects of metabolic health to dairy proteins *per se* is equivocal. Notably the relationship between increased intake of total protein from the diet and cardiometabolic endpoints is not without controversy (35, 36), in particular for T2D where some cohort studies show high total protein or animal protein intake to be associated with an increased risk of disease (37–39), including some proteins of dairy origin (40). Clearly protein source and quality is of considerable importance when determining dietary guidelines for prevention of T2D. Clinical evidence from RCTs regarding both dairy foods and dairy proteins point to wide ranging interventions reporting enhanced insulin secretion and associated endpoints, which in turn may result in better glycemic control. Wirunsawanya and colleagues, in their meta-analysis of whey protein from nine RCTs in overweight and obese referred to previously, showed significant improvement in fasting glucose albeit alongside a parallel decrease in body weight (30). Badely et al., also reported improvements in blood glucose in their systematic review of 37 published whey protein intervention trials in overweight and obese (32), but again whether this is independent of weight loss is not determined. This body of evidence is predominantly based on short duration studies of intermediary metabolic endpoints rather than long-term trials of incident T2D prevention, with long-term interventions of dairy protein on T2D endpoints or incidence lacking. Two trials planning to investigate moderate-term whey protein intervention for T2D management over a 3 months duration can be identified through review of international clinical trial registries. Almario et al. who have published acute effects (41) were unable to undertake their trial due to personnel changes [information kindly provided by the investigators; (42)]. Jakubowicz et al. (43) showed decrease in both postprandial glucose and HbA_{1c} on the high whey protein arm when compared with a low protein soy control. Notably, long-term dietary interventions for T2D prevention in overweight and high risk individuals to date are focused almost entirely on manipulation of the fat and carbohydrate components of the diet (44). The large European-led 2,500 participant, 3 years RCT, PREVIEW (PREVention of diabetes through lifestyle intervention In Europe and around the World) is the first long-term study to compare a novel higher total protein diet with a higher carbohydrate diet for incident T2D prevention (45). No significant improvement over the current best practice higher carbohydrate diet was found however. No long-term dairy protein RCTs investigating prevention of incident T2D have as yet been reported.

Acute intervention studies have provided some insights into potential mechanisms (46), with evidence that dairy proteins have more potent effects on insulin and incretin secretion compared to other commonly consumed animal proteins (21). Insulinotropic effects are associated with both the amino acid composition and the bioactive peptide profile, where for example inhibition of dipeptidyl peptidase-4 (DPP-4) increases incretin levels, which in turn inhibits glucagon release, increases insulin secretion and decreases gastric emptying (**Figure 1**). In concert, these in turn decrease circulating blood glucose concentrations. The amino acid content is driven in large part by the major contribution of whey protein to dairy, and consequent high levels of branched-chain amino acids (BCAAs) leucine, isoleucine and valine, as well as lysine (47). Clinical studies have reported dairy AAs, including leucine, isoleucine, glutamine, phenylalanine, proline and lysine, to have beneficial effects on glucose homeostasis. BCAAs stimulate the secretion of insulin and glucagon in addition to incretins glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) (47). In practical terms however, whether the dose of amino acids required to attain these protective responses can readily be achieved through recommended daily dairy intake is less well established (48). Clearly, more longer-term studies that can unravel the relationship between dairy protein and insulin and glucose control are needed.

In summary, there are no large RCTs evaluating effects of long-term dairy protein intake on either CVD or T2D incidence. There are however some preliminary conclusions to be drawn. Whilst cohort studies relating whey and casein *per se* to CV and T2D outcomes are lacking, RCTs have identified beneficial moderate-term effects of whey protein on intermediary risk factors including lipids and lipoproteins, blood pressure, and glucose-related parameters including insulin secretion. Acute postprandial studies provide evidence for whey protein and constituent AAs as promoting insulin secretion as a mechanism for improved glycemic control. Since much of the RCT evidence, however, has been obtained in overweight cohorts, the possibility that body weight and adipose mass loss may contribute to improvements in

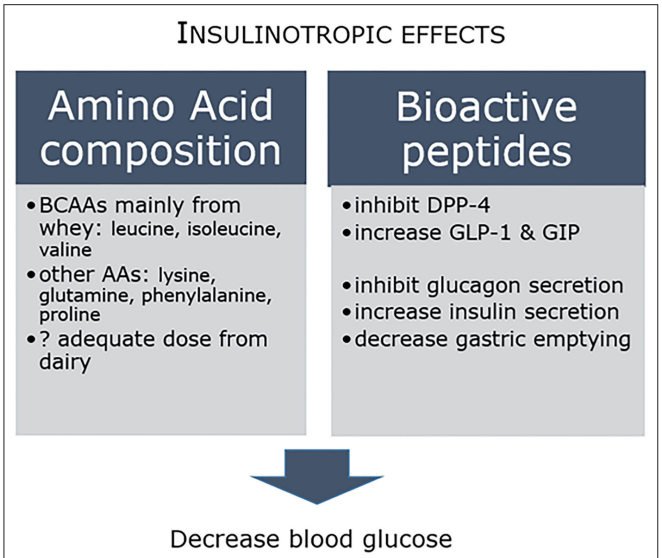

FIGURE 1 | Potential mechanisms of glucose lowering by dairy caseins and whey proteins.

metabolic health seen in these dairy intervention trials cannot be excluded.

Dairy Fats

The recent expert panel position paper referred to previously also reported on evidence underpinning relationships between dairy fatty acids and metabolic health. The panel concluded that medium-chain fatty acids/triglycerides (MCFA/MCT), odd chain fats, very long-chain SFAs and *trans*-palmitoleic acid were associated with lower T2D risk and improved metabolic health (18). They also noted, however, the possibility that findings were confounded by dairy fatty acids acting as markers of overall dairy consumption. Other evidence has been presented for milk fat globule membrane (MFGM) and polar phospholipids, *trans* vaccenic acid, and conjugated linoleic acid (CLA) in relation to both T2D and CVD risk, as summarized below.

Medium Chain Triglycerides (MCT)

MCTs are classified as lipids containing medium chain fatty acids of 6–12 carbon chain length. They are minor components of global diets but have long been investigated as a potential dietary lipid substitute for the more abundant long chain triglycerides (LCT). Milk is a reasonable source of saturated MCTs, comprising up to ∼15% of total lipid (22). MCTs undergo rapid hydrolysis and absorption, and suppress lipedema, hypothesized to predispose MCTs as cardioprotective (49). Evidence remains preliminary however. MCTs undergo almost complete hydrolysis to free fatty acids (FFA) and are absorbed directly into the portal vein, and hence transported rapidly to the liver for oxidation. This is in contrast to LCTs which are more slowly absorbed and transported by chylomicrons into the systemic circulation prior to oxidation or storage. The concept of rapid oxidation enhancing peripheral satiety signals and in turn promoting body weight control has been investigated in several studies, but with little underpinning evidence from human studies (50).

Evidence of enhanced metabolic health comes from several sources. *In vitro* studies of skeletal muscle cells show MCTs to enhance oxidative mitochondrial capacity and decrease lipid accumulation relative to LCTs, but not activate NF-κB or decrease insulin sensitivity (IS) (51, 52). Preliminary evidence also exists from *in vivo* studies, reporting ameliorated body fat accumulation (53) and insulin resistance in animals fed MCTs vs. saturated LCTs, which aligns with these prior studies. In the DairyHealth study, a 12 weeks cow's milk intervention conducted in Europe, dairy MCTs have been shown to induce gene expression of energy metabolism-related pathways in adipose tissue samples collected by biopsy from adults with abdominal obesity (54). These authors also showed a cardiometabolic-protective decrease in inflammation-related gene expression. Conversely, in animal models MCTs have also been shown to increase hepatic *de novo* lipogenesis and triglyceride accumulation, and adversely decrease hepatic IS, when compared with a lower fat diet (51, 53). Ameliorated lipid storage has also been reported in clinical studies of overweight adults, including following consumption of high-MCT dairy butter in combination with dairy protein (55). The DairyHealth study of abdominally obese adults also reported a higher intake of milk MCT to increase lean body mass and decrease total body fat % over 12 weeks, in an isoenergetic fat quality manipulation (56).

Odd Chain

The odd-chain fatty acids (OCFAs), pentadecanoic (C15:0) and heptadecanoic acid (C17:0), are SFAs that are found in dairy. Minor components that comprise ∼1.5% of milk fat, with C15:0 reported as approximately twice as abundant as C17:0. Long investigated as potential independent serum biomarkers of dairy intake (57, 58), these SFAs with odd number of carbons were proposed to be synthesized only by bacterial flora of ruminants (59, 60) with no metabolic precursors in humans. However, this has been challenged by emerging data of endogenous synthesis and metabolism of these OCFAs (61, 62). Adipose tissue depots rather than circulating fatty acids have more recently shown promise, with evidence that adipose C15:0 may reflect both habitual intake as well as changes in intake of dairy foods (63). With respect to health, recent reviews have reported that dairy OCFAs may be inversely associated both with cardiometabolic risk (20) and T2D (63) in cohort studies. C15:0 has also been proposed as an essential fatty acid (64) with some evidence in animal models of amelioration of inflammation and dyslipidemia.

Milk Fat Globule Membrane (MFGM) and Polar Phospholipids

The MFGM is the biological membrane that surrounds the lipid droplets in liquid milk. This and associated phospholipid (PL) components of milk continue to be associated with health benefits including those of metabolic health, as recently reviewed by Anto et al. (65) in a narrative review which presented 11 RCTs of milk PL effects on circulating serum lipids. A growing evidence base is proposed for conditions as varied as adverse lipid

metabolism (66–68), insulin resistance (69), inflammation (70), CVD through attenuated development of atherosclerosis (65), gut health (71) and neurodevelopment. Structurally the MFGM encases the fat globules within milk. It is comprised of proteins, cholesterol and polar rather than neutral lipids, including PLs such as phosphatidylcholine (PC) and sphingolipids such as sphingomyelin (SM). Recent mass spectral analysis of bovine milk has confirmed PC, phosphatidylethanolamine (PE) and SM as the most abundant polar lipid classes (72). The total polar lipid content of a product may vary greatly as a result of dairy processing, but typically comprise only ∼1% of the total lipid content of milk. Yet they contribute to several classical dairy food performance characteristics. The ability to stabilize oil-in-water emulsions is one of these, such that the polar lipids enable the emulsification of neutral triglyceride in the aqueous phase of liquid milk. When consumed in significant amounts within the diet, PLs can inhibit lipid absorption from the GI tract, acting mainly by altering mobilization of lipid from spherical micelles that form in aqueous solution (73). Disruption of the MFGM, such as during homogenization, is discussed in more detail in sections below.

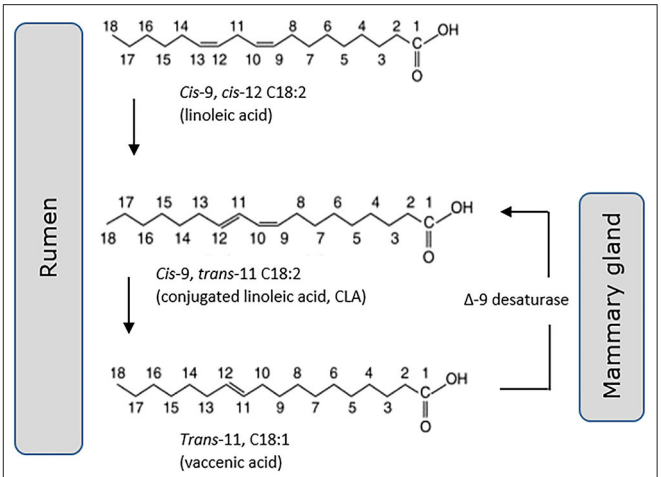

FIGURE 2 | *Cis*-9, *trans*-11 CLA (18:2) is generated both through hydrogenation of linoleic acid (18:2) in the rumen and desaturation of *trans*-11 vaccenic acid (18:1) in the mammary gland.

Trans-vaccenic Acid and *Trans*-palmitoleic Acid

Trans-11 vaccenic acid (C18:1, t11) is a monounsaturated "natural" *trans* fat, specific to ruminant-origin products of dairy and meat. There is some evidence (74), although not universal (75, 76), that it lacks the adverse cardiometabolic health outcomes associated with other forms of dietary *trans* fats, long developed by the food industry through industrial lipid hydrogenation. There are a number of *in vivo* and *in vitro* reports of positive effects of dairy *trans* fats on metabolic endpoints including increased insulin secretion and pancreatic islet β-cell growth (77), decreased hyperlipidemia (78), and ectopic liver fat accumulation (79). These reports however must be balanced against others that do not support a health benefit. A 2010 systematic review of 39 clinical intervention trials by Brouwer et al. (75) included 29 industrial *trans* fats, 17 CLA and six ruminant *trans* fats studies. They concluded that all fatty acids with a *trans* double bond raised the LDL- to HDL-cholesterol ratio in an adverse manner. In an update of this review they noted the contradictory evidence that observational studies fail to demonstrate a higher risk of CVD associated with high intake of ruminant *trans* fats, despite confirming adverse effects on circulating blood lipid profile (80). Conversely, evidence of positive outcomes for T2D has been building, where monounsaturated *trans*-palmitoleic acid (16:1, t9) is of particular interest. Associated in recent publications with a lower incidence of T2D (18, 81) and coronary artery disease (CAD) (81), it is both consumed within dairy as well as delivered through endogenous conversion from its metabolic precursor *trans*-vaccenic acid (82). Evidence for a protective effect of dairy *trans*-palmitoleic comes from a recent large meta-analysis of 16 prospective cohort studies comprising more than 60,000 participants. Conducted by the fatty acids and outcomes research consortium (FORCE) this analysis showed a significant association of *trans*-palmitoleic with a lower risk of T2D (58). A note of caution must be added however, with several authors questioning the robust nature of methods used for analysis of fatty acid methyl esters (FAMEs) from bovine milk, citing difficulties in full separation and unequivocal identification of FAMEs within the very complex lipid composition of ruminant milk (83, 84). The various pathways linking these *trans* fats to CLA are now reasonably well understood, including desaturation of *trans*-11 vaccenic acid (18:1) by the Δ9-desaturase enzyme within the mammary gland to form *cis*-9, *trans*-11 CLA (18:2) (**Figure 2**). This is in addition to formation of CLA via biohydrogenation of linoleic acid (18:2) within the bovine rumen, and in turn synthesis of *trans*-vaccenic. Synthesis via Δ9-desaturase has been proposed to be the primary source of *cis*-9, *trans*-11 CLA in bovine milk fat (85).

Conjugated Linoleic Acid (CLA)

CLA is a polyunsaturated fatty acid (PUFA) with conjugated double bonds found in both *cis* and *trans* forms, the most common isomer of which is *cis*-9 *trans*-11 linoleic acid. Despite being found in ruminant dairy and meat, and in lesser amounts in plant oils and seafood, CLA is also widely produced industrially for sale as a supplement. Much like vaccenic acid, there are reports of cardiometabolic health benefits (86, 87). A systematic review and meta-analysis of 33 RCTs that investigated the effect of ruminant CLA, in either supplement form or from enriched foods, on lipid profile in healthy adult populations reported that both formats significantly decreased LDL-C with minor non-significant effects on other lipid outcomes (87). The review also however noted concerns on potential safety of CLA with reports of increased insulin resistance and lipodystrophy in animal models, in addition to some adverse lipid and C-RP responses in clinical studies. In line with this note of caution, several recent reviews have concluded that many positive outcomes reported in animal models or *in vitro* have not been adequately replicated in clinical trials (88, 89). The 2018 review by Li et al. reported the lack of clinical data for hard CV endpoints. They identified only 1 observational case-control study showing an inverse relationship

between levels of cis-9, *trans*-11 CLA in adipose tissue and the risk of heart attack (90), and 3 RCTs all showing no significant effect of CLA on blood lipids, glucose, CRP, blood pressure, insulin resistance, body composition or 10-year absolute risk of fatal CVD in obese patients (91). There was a similar lack of efficacy reported in healthy cohorts (92) and patients with atherosclerosis (93). There is evidence of improved C-RP levels from one intervention trial in patients with active CVD (94). Conversely there is evidence, as for vaccinic acid, that CLA adversely affects lipoprotein profile, with increased circulating LDL-cholesterol and total:HDL-cholesterol ratio in an exhaustive review of 48 CLA and 11 ruminant *trans* fat RCTs (80). In addition, worsening of associated CVD risk factors including the acute phase protein C-RP [Haghighatdoost and Nobakht-M-Gh 2018, (95)] and cytokine TNF-α have been identified in a recently published systematic review (96). In addition, a 2017 meta-analysis of 32 RCTs of food-enriched and supplement CLA has also shown no significant effect on fasting glucose (88). A recent narrative review has noted lack of consensus interpreting effects of CLA on body weight loss and obesity due to small sample size, variable dose, variable CLA isomers, variable intervention durations and population characteristics (97); but that beneficial effects of supplementation on parameters related to body weight and/or adiposity have been reported in a number of trials, with no evidence of adverse metabolic consequences. This review also reports similar absence of consensus for glycemic endpoints, with both beneficial glucose metabolic effects and no clinical benefit reported in RCTs (97).

In summary, there are no RCTs evaluating long-term effects of these specific dairy fat components on either CVD or T2D incidence. Very little evidence supports MCTs as cardioprotective, with preliminary clinical findings related only to modest improvements in body composition. In turn, OCFAs have been associated with decreased CV and T2D risk in cohort studies, but no intervention data is available. Conversely, there is limited evidence that MFGM and the associated PL components of dairy may improve CV and T2D intermediary endpoints, in RCTs of 1 day to 12 weeks duration reporting outcomes including lipid metabolism, inflammatory response and insulin resistance. The evidence that underpins *trans*-fats is more complex and controversial. Ruminent-origin *trans* vaccenic acid (*trans*-VA) may not generate the well-demonstrated adverse response in CV risk endpoints of industrial *trans*-fats. There is lack of concordance in the evidence base however, with proposed neutral effects of *trans*-VA observed in cohort studies not being supported by RCT evidence, where systematic review has concluded that all fatty acids containing a *trans* double bond adversely increase lipoprotein profiles, irrespective of their origin. Conversely there are reported positive T2D risk outcomes for the downstream product of *trans*-VA, *trans*-palmitoleic acid (*trans*-PA) which is also present in minor quantities in dairy, and which cohort studies have correlated with decreased T2D risk. CLA has also been proposed as cardioprotective, with both ruminant-origin and industrial-origin supplements found within the western diet, and highly variable levels of evidence published. From meta-analysis of RCTs showing both significant improvement and significant worsening in lipoprotein risk factors, alongside no significant effects on FPG, to very limited data supporting an association with improved CV incidence in observational studies. There remains no current consensus on outcomes likely due to factors including the highly variable isomers and dose of CLA consumed, size and type of experimental cohorts recruited, and duration of the RCTs.

Micronutrients: Vitamin D, Calcium

Relationships between vitamin D, calcium, and various aspects of cardiometabolic health have long been controversial. In this review, focus has been given to the evidence underpinning relationships between vitamin D and T2D, and calcium and CVD. Dairy naturally contains little vitamin D but is a food category much debated with respect to fortification. Fluid milk and its myriad products have a history of both mandatory or voluntary fortification with vitamin D by the food industry. A recent review reported milk products currently to be systematically, either mandatory or voluntary, fortified with vitamin D only in European Nordic countries of Finland, Norway, Sweden as well as in North American countries of Canada and the United States (98).

There has been conflicting observational evidence for a number of years which has linked low levels of 25-hydroxyvitamin D (25(OH)D) to T2D, and has led to vitamin D replacement interventions conducted with intent to both improve glycemic control and decrease T2D incidence (99). Both synthesized in the skin in response to sunlight exposure and/or consumed within the diet and as a supplement, 25(OH)D is the main circulating form of vitamin D. It has been linked to dysglycaemia and T2D through mechanisms that affect both insulin resistance and pancreatic β-cell insulin secretion, as well as inflammation and accumulation of advanced glycation end (AGE) products (**Figure 3**). A number of early systematic reviews and meta-analyses supported the protective inverse relationship between vitamin D and T2D. An analysis of 8 observational cohort studies reported that high vitamin D status (>25 ng/ml) was associated with an almost halving of risk of T2D compared with low status (100). Another more recent review of 21 cohort studies showed a similar relative risk of 0.62 and using linear trend analysis also reported that for each 10 nmol/L increment in circulating 25(OH)D levels there was an associated 4% lower risk of T2DM (101). A further meta-analysis of 16 cohort studies also showed a positive outcome (102). Adiposity and adipose mass is a confounding factor in the relationship between vitamin D and T2D. Vitamin D is sequestered in adipose tissue, with the greater adipose mass reported to act as a "reservoir" for vitamin D, and the increased amount of vitamin D required to saturate the large depots of obese individuals predisposing to low levels of circulating 25(OH)D (103). In turn obesity has long been identified as a very strong predisposing factor for T2D (104).

Far fewer clinical intervention trials have been conducted, with confounding issues including failure to reach sufficient circulating levels of vitamin D (105). Required circulating levels of 25(OH)D necessary to effect glycemic control and decrease risk of developing T2D, have not yet been determined. It is speculated that these may be higher than that required for

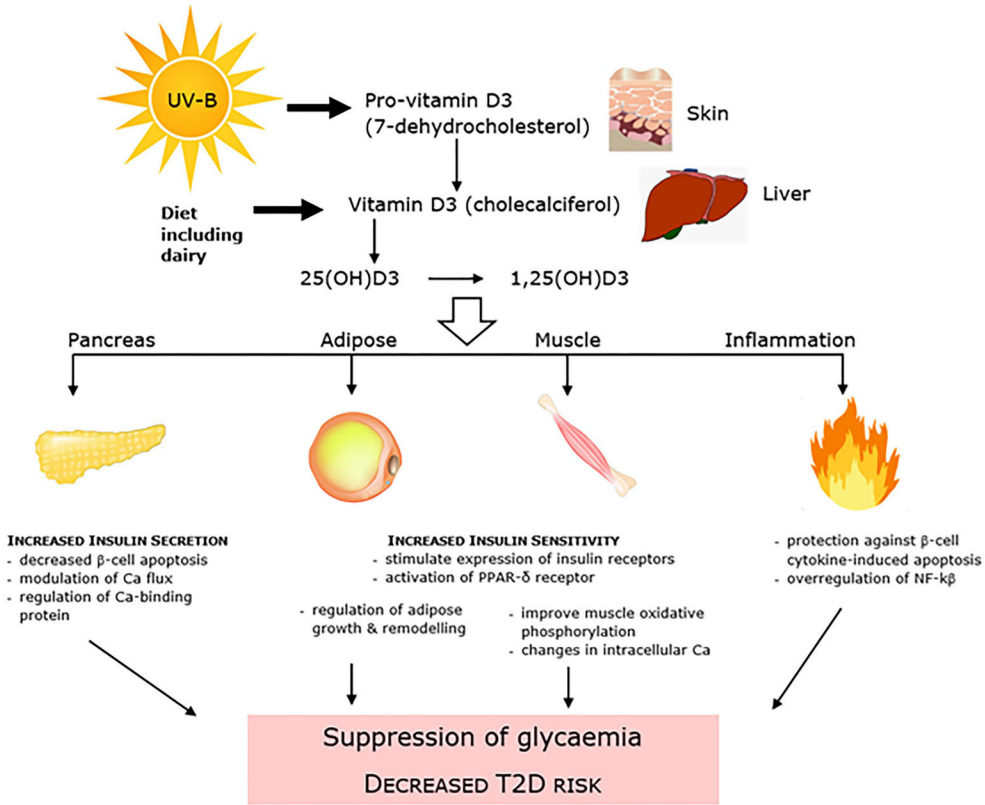

FIGURE 3 | Vitamin D is linked to dysglycaemia and T2D through mechanisms that affect both pancreatic β-cell insulin secretion and insulin resistance, as well as systemic inflammation.

optimal bone health (99). Conclusions have varied widely from meta-analyses that report positive cardiometabolic outcomes, including findings from an analysis of 9 RCTs which showed that vitamin D-fortified yogurt decreased HOMA-IR, fasting serum glucose and total cholesterol in normoglycaemic and T2D patients (106), and from analysis of 19 RCTs which showed improvements in both IR and HbA_{1c} in T2D patients (107). Conversely prior systematic reviews reported no effect of vitamin D supplementation on glycemic outcomes in a meta-analysis of 11 (100), and 15 RCTs of normoglycaemic and T2D patients. The latter however showing that sub-cohort analysis of T2D or IGT patients resulted in a small effect on fasting glucose and IR, but not HbA_{1c} (108).

Calcium and metabolic health has been under considerable scrutiny in recent years. Overall, calcium is not a convincing contributor to cardiometabolic benefits that may be seen from dairy. Commonly used as a supplement to promote bone health with contributions to prevention of fracture risk in the elderly, meta-analysis of long-term RCTs unexpectedly identified calcium supplementation to result in moderately increased risk of heart attack (109, 110). The mechanism that may underpin this risk has been proposed to be postprandial hypercalcemia, in turn contributing to vascular calcification. Interestingly, single-nucleotide polymorphisms (SNPs) related to higher serum calcium levels have more recently been associated with increased risk of coronary artery disease and myocardial infarction in genome wide studies (111). It has become clear, however, that it is important to differentiate the origin of the calcium consumed, with different effects between calcium consumed as a dietary supplement vs. calcium consumed as a food from dairy sources. Calcium supplements, but not calcium consumed within food products, have been proposed to result in a negative risk-benefit, with routine use for prevention or treatment of osteoporosis no longer universally recommended (112). Indeed, current evidence no longer supports routine calcium or vitamin D supplementation for bone health in healthy adults resident within the community, based in major part on the adverse CVD outcomes (113).

The evidence in support of dairy-origin calcium for T2D prevention is also mixed, although as recently reviewed (22), there are multiple mechanisms by which calcium may influence T2D risk factors. This includes regulation of insulin-mediated intracellular processes in specific tissues that respond to this peptide, contribution to secretory function of β-cells within the pancreas, and phosphorylation of insulin receptors. Calcium has also been shown to down-regulate genes encoding pro-in?ammatory cytokines that are involved in IR. In their review Mozaffarian et al. also reported >20 observational studies on associations between calcium intake and T2D prevention (22), of which 3 large cohort studies in US and China showed an

inverse association between high calcium intake and low T2D risk in subcohorts of women but not men. A 4th large cohort study in Japan also observed this inverse relationship but only in subcohorts with higher serum vitamin D. The other smaller studies reported mixed outcomes, with no clear consensus. Additional to this review, the Korean Genome and Epidemiology study [KoGES (114)], a prospective cohort trial followed for 10 years, reported the positive outcome that higher dietary calcium, but not serum calcium, was associated with a lower risk of developing T2D in a study of >10,000 adults from an Asian cohort.

Processing: Fermentation, Probiotics, and Homogenization

There is considerable evidence that processing methods including fermentation, addition of probiotic bacteria and yeasts, and homogenization can all influence cardiometabolic health, as presented in the sections below.

Fermentation

Both cheese ("cultured") and yogurt are commonly consumed fermented dairy products. The fermentation process requires a starter culture, which may vary greatly in order to achieve required texture, flavor and safety profile. Lactic acid bacteria are the major bacteria used in the fermentation process of modern food products, and can include the genera *Lactobacillus, Lactococcus, Streptococcus, Leuconostoc, Pediococcus*, and *Enterococcus*, whilst yeasts and molds are also used in cheese production (115). Acidification rate through production of lactic acid in addition to the secretion of secondary metabolites, including bacteriocins, biogenic amines, exopolysaccharides, and proteolytically released peptides, are key aspects of the fermentation process. Notably, neither cheese nor yogurt exert the adverse effects on blood lipids that would be predicted solely by the content and composition of their component SFAs (116, 117). Consumption of yogurt is clearly associated with decreased risk of both T2D and cardiometabolic disease, as reviewed by the recent expert panel of Guo et al. (18). Whilst a number of factors may contribute, the fermentation processes and the probiotic content and composition have both been proposed as significant contributors.

Probiotics

Probiotic bacteria are common components of fermented foods, including dairy set and drinking yogurts and the fermented dairy drink kefir. Kefir, similar to a liquid drinking yogurt, is a more recent introduction to the wider Westernized diet, prepared through inoculation of ruminant milk with kefir "grains," a product which contains both bacteria and yeasts. Multiple probiotic genera have been evaluated in cohort studies and RCTs, with the two most common being *Lactobacillus* and *Bifidobacterium*. Probiotics are long proposed to have positive health effects achieved through alterations in both composition and function of the host large bowel microbiome, and there is a large literature investigating obesity-related metabolic health outcomes (118). Mechanisms proposed include improved intestinal epithelial integrity decreased low grade endotoxemia-induced inflammatory response, and promotion of colonic short chain fatty acids (SCFA) including butyrate as a nutrient source for the large bowel colonocytes (119, 120). It has been reported both that probiotic effects of yogurt may modulate glycaemia and related endpoints, and exert beneficial effects (117), or conversely that the evidence is lacking. Astrup evaluated both cohort studies and RCTs, concluding that observational data supported the significant association between yogurt, decreased risk of body weight gain, and CVD, but that notably this evidence was supported only in part by RCTs. A later 2017 review that reported on 7 RCTs, in addition to animal model evidence, in turn also concluded that the effects of probiotics on glycemic control were conflicting (121). A 2016 narrative review (122) of probiotics, prebiotics and combination symbiotic RCTs concluded that probiotics have only a small (∼3%) effect on metabolic endpoints, with outcomes maximized when consumed within fermented milks or yogurts over an extended period of at least 8 weeks.

An important question is whether there is an independent causative relationship between probiotics *per se* and improvements in CVD, T2D or other metabolic health outcomes, independent of the food matrix such as yogurt. Notably, a recent systematic review and meta-analysis of RCTs evaluated the effects of probiotic yogurt vs. control yogurt on glycemic outcomes in obese individuals and those with T2D and reported no additional benefits of the probiotic yogurt (123). A comprehensive recent systematic review and meta-analysis of 20 prospective cohort studies and 52 RCTs (124) has highlighted the positive effects of fermented milks, yogurts and probiotics. The review reported fermented milk consumption to be associated with decreased CV risk, with probiotics added into dairy matrices lowering serum lipid profile, while yogurt consumption was associated with decreased T2D and metabolic syndrome risk. The authors did note however that heterogeneity of these multiple studies including variability in probiotic strains requires the outcomes to be interpreted with caution.

Homogenisation

Cow's milk is commonly homogenized in order to increase the physical stability of the food product. It aids in the prevention of separation of water and lipid components into bilayers through a decrease in lipid droplet size and incorporation of dairy protein into the droplet interface. The decrease in milk fat globule size, essentially a disruption in the MFGM, which allows them to be dispersed uniformly through the milk product, has been proposed to result in improved digestibility and potentially health outcomes (125). The recent review by Mozaffarian et al. (22) noted that homogenization may destroy MFGM, and proposed that this may have implications for the cardiometabolic effects of dairy lipids. They cited an 8 weeks RCT which compared whipping cream (intact MFGM) with energy-, total dairy- and SFA-matched butter fat (homogenized, decreased MFGM) (67). Butter fat worsened both LDL-C and apolipoprotein B:A-I ratio, with no effects during whipping cream intervention.

DAIRY AND THE SATURATED FAT CONTROVERSY

The high saturated fatty acid (SFA) content of dairy and its myriad of food products has long raised concern (126), aligned with international guidelines (126, 127) to minimize dietary intake of animal-origin SFAs to achieve better cardiometabolic health. Early systematic reviews supported a relationship between dietary fatty acids and serum lipids, and also serum lipids and CV morbidity and mortality, as recently summarized by Hooper et al. (128). Mechanisms by which animal-origin SFAs may adversely affect circulating lipids include inhibiting LDL-receptor removal of lipoproteins from circulation, enhancing secretion of ApoB100-containing lipoproteins VLDL and LDL into circulating, and packing excess cholesterol in LDL particles; hence in combination increasing circulating LDL-C, TC and LDL-C:HDL-C ratio concentrations. In their Cochrane systematic review these authors in turn have shown a prolonged (2 years+) decrease in dietary SFA to be associated with a significant decrease in combined CV events, with some evidence of a dose-relationship (128). Replacing SFA with PUFA, but possibly not MUFA, and/or carbohydrate has been proposed as the most successful approach. However, over the past 10 years a consensus of studies has built which has questioned the assumption of adverse effects for dairy-origin SFAs for both CV and T2D outcomes. Notably, it is no longer adequate to consider nutrients in isolation, with evidence that the complex matrix of a food may be equally or more important than the fatty acid content and composition alone when predicting cardiometabolic risk (3, 10, 11). It has been proposed that in a complex dairy food such as cheese, for example, the effect of SFAs on blood lipids and disease risk may be counterbalanced by the content of protein, calcium, or other dietary components (23, 129). Aside growing evidence that dietary SFA-induced increases in LDL-cholesterol may not *per se* adversely affect cardiometabolic health (3).

Evidence that underpins the relationship between dairy fat and cardiometabolic outcomes to date exists predominantly from observational studies. This must be noted since association studies cannot determine cause and effect relationships, and are subject to confounding that may bias relationships between diet and disease outcomes. Human clinical trials investigating dairy interventions characteristically have been of short- or moderate-duration, with CV or T2D intermediary risk endpoints, and not disease incidence, evaluated. A summary of both is presented below.

Observational Studies

CVD: Contrary to prior concerns for high SFA animal-origin food groups (1), there is no consistent evidence from epidemiologic studies that a higher intake of dairy products is associated with increased CVD risk or incident CVD (3, 4, 6, 7, 20, 130). Key systematic reviews, meta-analyses, umbrella and narrative reviews are presented in **Table 1a**. In 2014 Astrup summarized observational studies which reported that the consumption of milk or dairy products was in fact inversely related to incidence of CVD (117), an outcome supported in a recent state of the art review (3). Inclusion of dairy products in the diet may ameliorate characteristics of adverse metabolic health including dyslipidemia, insulin resistance, hypertension, abdominal obesity, and the metabolic syndrome cluster (137), which together markedly increase the risk of both T2D and CVD. This had been proposed previously in meta-analyses of prospective cohort studies that identified an inverse association between milk and other dairy components and CV endpoints (4, 5, 116), with Elwood et al. originally noting a mismatch between this evidence and perceptions of harm from consumption of dairy (4). In 2015 a meta-analysis of prospective cohort studies provided further evidence supporting the beneficial effect of dairy consumption on CVD. Low-fat dairy products and cheese may protect against stroke or CHD incidence (6), in particular yogurt (134). A 2016 meta-analysis of prospective cohort studies again showed that dairy consumption may be associated with reduced risks of CVD, albeit with the authors noting that additional data are needed to more comprehensively examine potential dose-response patterns (7). It is now apparent that most meta-analyses report no association or even a weak inverse association between intake of dairy products and CVD endpoints (8, 20, 131, 133, 135, 136), or risk biomarkers including serum lipoprotein LDL-cholesterol. Characterized as convincing probable evidence, a recent 2020 umbrella review by Godos et al., reported a decreased association between total dairy and CVD (9). Arguably there is more controversy with respect to butter fat studies (2, 132). The recent large multinational Prospective Urban Rural Epidemiology (PURE) study from 21 countries in five continents has since also reported similar findings, with dairy intake associated with lower risk of mortality and major CVD events (130). However, even in light of the absence of a positive adverse association between dairy and CVD, there is some evidence that replacing dairy fat with PUFAs, especially from plant-origin foods, may be advantageous and confer positive health benefits (20).

T2D

Whilst prospective cohort studies have commonly shown a range of animal products, for example red and processed meats, to be associated with increased risk of T2D (138), conversely numerous meta-analyses and systematic reviews report dairy food consumption to be linked to neutral or even decreased T2D risk (see **Table 1b**). These reviews utilize data from adult populations globally, reporting greatest effects with yogurt and cheese (19), in turn representative of both low-fat and high-fat dairy, respectively. The early meta-analysis of observational cohort studies showed higher total dairy also to be associated with a reduction in T2D risk (4), as did another small meta-analysis of 7 cohort studies (12). In 2013 Aune et al. published a meta-analysis of 17 prospective cohort studies, reporting that a higher consumption of total dairy products and cheese, as well as low-fat dairy, was associated with a lower risk for T2D (13). Further, investigation of dose-response showed a 7% decrease in T2D risk for every 400 g total dairy food consumed per day, which was estimated to be equivalent to ∼1.7 servings of liquid milk per day (13). A second meta-analysis of 14 prospective cohort studies confirmed a similar 6% decrease in T2D risk for every 200 g of total dairy per day, plus in turn a 12% decrease for

TABLE 1a | Summary of meta analyses, systematic reviews and narrative reviews reporting CVD outcomes: observational studies.

References	No. of studies	Study design	Dairy formats	Outcomes
Elwood et al. (4)	Eleven studies prospective cohort	Meta-analysis	Total dairy, milk, butter, cheese	Inverse association for total dairy vs. IHD RR 0.92 (0.80, 0.99) and stroke RR 0.79 (0.68, 0.91). Mismatch between evidence from long-term prospective studies and perception of harm from consumption of dairy
Soedamah-Muthu et al. (5)	Seventeen studies Prospective cohort	Meta-analysis	Total dairy, low-fat, high-fat dairy	Modest inverse association for milk and CVD (4 studies) RR 0.94 per 200 mL/d, 95% CI: 0.89, 0.99; but not CHD (6 studies) RR 1.00, 95% CI: 0.96, 1.04; or stroke (6 studies) RR 0.87, 95% CI: 0.72, 1.05. No association for high-fat or low-fat dairy, per 200 g/d, with CHD.
Huth and Park (116)	Twenty-three studies Prospective cohort	Narrative review	Total dairy, low-fat, high-fat, milk, cheese, butter, yogurt	Most, but not all, showed no relationship or inverse association between dairy and risk of CVD and stroke
Astrup et al. (117)	Seventeen studies Prospective cohort	Narrative review	Milk, yogurt, mixed dairy	Modest inverse association between milk and risk of CVD, RR reduction 6%. Milk not associated with reduction in risk of CAD, stroke, or total mortality.
Qin et al. (6)	Twenty-two studies Prospective cohort	Meta-analysis	Total dairy, milk, low-fat dairy, high-fat dairy, cheese, butter, yogurt	Inverse association for total dairy and CVD (nine studies; RR 0.88, 95% CI: 0.81, 0.96), stroke (12 studies; RR 0.87, 95% CI: 0.77, 0.99), but not CHD
Alexander et al. (7)	Thirty-one studies Prospective cohort	Systematic review and meta-analysis	Total dairy, dairy products, low-fat dairy, high-fat dairy, Ca from dairy	Inverse association for total dairy (RR 0.91; 95 % CI 0.83, 0.99) and calcium from total dairy (RR = 0.69; 95 % CI 0.60, 0.81) and stroke; also cheese and CHD (RR 0.82; 95 % CI 0.72, 0.93) and stroke (RR 0.87; 95 % CI 0.77, 0.99). Low level evidence after adjustment for within-study covariance
Drouin-Chartier et al. (131)	Twenty-one studies Prospective cohort	Systematic review and meta-analysis	Total dairy, milk, high-fat dairy, low-fat dairy, cheese, yogurt, fermented	Favorable or neutral associations with CVD-related clinical outcomes. Moderate-quality evidence for total dairy as neutral for CVD risk High-quality evidence for total dairy and decreased hypertension risk
Pimpin et al. (132)	Fifteen studies Prospective cohort	Systematic review and meta-analysis	Butter fat	Not significantly associated with CVD
Guo et al. (133)	Twenty-nine studies Prospective cohort	Meta-analysis	Total dairy, low-fat dairy, high-fat dairy, milk	No associations for total (high-fat/low-fat) dairy, and milk with CHD or CVD
Wu and Sun (134)	Nine studies Prospective cohort	Meta-analysis	Yogurt	Highest category consumption ns related to incident CVD; RR 1.01, 95% CI 0.95, 1.08
Gholami et al. (8)	Twenty-seven studies Prospective cohort	Meta-analysis	Total dairy	Inverse association between total dairy and CVD; no relationship for CHD
Dehghan et al. (130)	PURE study	Prospective cohort	Whole-fat dairy: milk, yogurt, cheese	Inverse association between higher dairy and CV events HR:0.81; 95% CI:0.77, 0.93
Yu and Hu (20)	Four studies Prospective cohort	Narrative review, summarizing meta analyses of cohort studies for CVD endpoints	Total dairy, milk, cheese, yogurt	Null or weak inverse association between consumption of dairy products and risk of CVD. Milk RR 1.01, cheese RR 0.92, yogurt RR 1.03. Odd chain fats as biomarkers: inverse association of C15:0 and C17:0 with CVD
Guillocheau et al. (81)	Thirteen studies Prospective cohort	Narrative review of cross-sectional, case control, cohort studies	Monounsaturated *trans*-palmitoleic acid	Neutral or inverse associations; but inconsistent findings for CVD risk factors
Hirahatake and Astrup (135)	Seventeen studies Prospective cohort	Narrative review summarizing meta-analyses, systematic reviews, prospective cohort studies	Multiple dairy formats including full fat, low-fat, cheese, yogurt	Neutral or inverse association of full-fat dairy with CVD
Kim et al. (136)	Sixty-two studies Korean cohorts; 42 cross-sectional, 3 case-control, 17 cohort	Systematic review and meta-analysis	Total dairy, milk	Inverse association with CVD risk factors
Godos et al. (9)	Fifty-three studies Prospective cohort	Umbrella review of meta analyses	Total dairy, milk, high-fat dairy, low-fat dairy, cheese, butter, yogurt	Inverse association for total dairy and CVD; and for cheese and CHD, CVD and stroke risk factors in highest vs. lowest category; no association with milk

CAD, coronary artery disease; CHD, coronary heart disease; CI, confidence interval; CVD, cardiovascular disease; HR, hazard ratio; IHD, ischemic heart disease; RR, relative risk.

TABLE 1b | Summary of meta analyses, systematic reviews and narrative reviews reporting T2D outcomes: observational studies.

References	No of studies	Study design	Dairy formats	Outcomes
Elwood et al. (4)	Five studies Prospective cohort	Meta-analysis	Total dairy, milk	T2D incidence 3.9%. Inverse association for total dairy and incident T2D in highest relative to lowest intake (RR 0.85; 95% CI 0.75, 0.96)
Tong et al. (12)	Seven studies Cohort	Meta-analysis	Total dairy, milk, high-fat dairy, low-fat dairy, yogurt	T2D incidence 4.0%. Inverse association for total dairy (RR 0.86; 95% CI 0.79, 0.92), low-fat dairy (RR 0.82; 0.74, 0.90), whole milk (RR 0.95; 0.86, 1.05), yogurt (RR 0.83; 0.74–0.93) and T2D; neutral for high-fat dairy (RR 1.00; 0.89, 1.10) and T2D
Aune et al. (13)	Seventeen studies Prospective cohort	Systematic review and meta-analysis	Total dairy, milk, high-fat dairy, low-fat dairy, yogurt	T2D incidence 5.7%. Inverse association for total dairy (400 g/d; RR 0.93; 95% CI 0.87, 0.99), high-fat dairy (200 g/d; RR 0.98; 0.94, 1.03), low-fat dairy (200 g/d; RR 0.91; 0.86, 0.96), milk (200 g/d; RR 0.87; 0.72, 1.04), cheese (50 g/d; RR 0.92; 0.86, 0.99), yogurt (200 g/d, RR 0.78; 0.60, 1.02) and T2D
Gao et al. (14)	Fourteen studies Prospective cohort and case control	Systematic review and meta-analysis	Total dairy, milk, high-fat dairy, low-fat dairy, yogurt	T2D incidence 5.6%. Inverse association for total dairy (200 g/d; RR 0.94; 95% CI 0.91, 0.97), low-fat dairy (200 g/d; RR 0.88; 0.84, 0.93), cheese (30 g/d; RR 0.80; 0.69, 0.93), yogurt (50 g/d; RR 0.91; 0.82, 1.00) and T2D
Chen et al. (15)	Fourteen studies Prospective cohort	Meta-analysis	Total dairy, milk, high-fat dairy, low-fat dairy, yogurt	T2D incidence 7.8%. Neutral association for total dairy (1 serve/d; RR 0.98; 95% CI 0.96, 1.01), inverse association for yogurt (1 serve/d; RR 0.82; 0.70, 0.96) and T2D
Gijsbers et al. (16)	Twenty-two studies Prospective cohort	Meta-analysis	Total dairy, milk, high-fat dairy, low-fat dairy, yogurt	T2D incidence 7.4%. Neutral or inverse association for total dairy (per 200 g/d, RR 0.97; 95% CI 0.95, 1.00), low-fat dairy (per 200 g/d; RR 0.96; 0.92, 1.0), yogurt (per 80 g/d; RR 0.86; 0.83, 0.90), ice cream (per 10 g/d; RR 0.81; 0.78, 0.85) and T2D
Pimpin et al. (132)	Eleven studies Prospective cohort	Meta-analysis	Butter	T2D incidence 3.8%. Inverse association for butter (RR 0.96; 95% CI 0.93, 0.99) and T2D
Drouin-Chartier et al. (131)	Seven studies Prospective cohort	Systematic review of meta analyses	Total dairy, high-fat dairy, low-fat dairy, dairy fat, milk, cheese, yogurt, fermented	High-quality evidence for inverse association of low-fat dairy and yogurt; moderate-quality evidence for total dairy and cheese; high to moderate evidence for high-fat dairy, milk, fermented dairy and T2D
Schwingshackl et al. (139)	Twenty-one studies Prospective cohort	Systematic review and meta-analysis	Dairy as food group; total dairy, high-fat dairy, low-fat dairy	T2D incidence 7.8%. Inverse association with total dairy (RR 0.91; 95% CI 0.85, 0.97) in highest vs. lowest category, but only in Asian and Australian not American and European studies; borderline inverse association for low-fat dairy, no association for high-fat dairy and T2D
Riserus and Marklund (63)	Nine studies Prospective cohort	Narrative review	Circulating dairy odd chain dairy fatty acids (OCFA), C15:0, C17:0	Inverse association of OCFA biomarkers with T2D, but not CVD; may reflect high-fat dairy only
Soedamah-Muthu and de Goede (140)	Twenty-six studies Prospective cohort	Systematic review and meta-analysis	Total dairy, low-fat dairy, yogurt	T2D incidence 0.8%. Inverse association total dairy (RR 0.97; 95% CI 0.95, 1.00), low-fat dairy (RR 0.96; 0.92, 1.00), yogurt (80 g/d RR 0.86; 0.83, 0.90).
Alvarez-Bueno et al. (17)	Twelve studies Cohort and case control	Systematic review of meta analyses	Total dairy, low-fat dairy, milk, yogurt	T2D incidence 7.4–7.8%. Inverse association for total dairy (RR range 0.86–0.91), low-fat dairy (RR range 0.80–0.83), low-fat milk (RR 0.82), yogurt (RR range 0.74–0.86), and T2D
Godos et al. (9)	Fifty-three studies Prospective cohort	Umbrella review of meta analyses	Total dairy, milk, high-fat dairy, low-0 fat dairy, cheese, butter, yogurt	"Possible" (evidence level) inverse association of total dairy, cheese, butter, yogurt; no significant association for milk and T2D
Imamura et al. (58)	Sixteen studies Prospective cohort	Meta-analysis	Circulating or adipose tissue dairy odd-chain fatty acids (OCFA) C15:0, C17:0, *trans*-palmitoleic acid	T2D incidence 23.8%. Inverse association for C15:0 HR 0.80; 95% CI, 0.73, 0.87; C17:0 HR 0.65; 0.59, 0.72; *trans*16:1-n7 HR 0.82; 0.70, 0.96; sum OCFA HR 0.71; 0.63, 0.79, and T2D

CI, confidence interval; HR, hazard ratio; IHD, ischemic heart disease; OCFA, odd chain fatty acids; RR, relative risk; T2D, type 2 diabetes.

every 200 g low-fat dairy per day (14). This increased further to a 20% lower risk in T2D for every 30 g of cheese, and a 9% lower risk associated with consuming 50 g per day of fermented yogurt showing an inverse association between dairy intake and risk of T2D. Gijsbers et al., meta-analysis also reported an inverse association between dairy intake and risk of T2D (16), as did Schwingshackl et al. (139) and Soedamah-Muthu et al. (140). An updated recent 2019 meta-analysis of 12 studies has also reported total dairy consumption to be associated with lower risk of T2D, with moderate effects of cheese and strong effects of yogurt and low-fat dairy (17), in supported by a 2020 review of 12 prospective cohort studies (9). Notably however, in a combined analysis of men and women from the US Health Professionals Follow-Up Study and the US Nurses' Health Study I and II, no relationships were observed with total dairy, low-fat dairy or high-fat dairy, with only yogurt intake associated with a decreased risk of T2D (15). Also contrary to earlier findings of a significant association with total dairy in these large US cohorts. The authors proposed the longer follow up (+10 years) may account for this change in outcome, in turn observed in a meta-analysis of prior cohort studies published by their research team where benefits of dairy intake were diminished in long follow up studies (15). Pimpin et al. reported an inverse association with incidence of T2D in their more recent meta-analysis of butter fat (132).

Other individual large prospective cohort studies in the US support a neutral, not adverse, relationship between total dairy and risk of T2D including postmenopausal women from the Women's Health Initiative Observational Study. Whilst higher dairy food consumption was associated with 40–50% reduced risk, it was notable that these large effects were related to low-fat dairy and yogurt intake (141). Prospective cohort studies conducted outside the US have also found beneficial or neutral effects of total dairy food consumption, as well as for specific dairy food groups which include high-fat dairy and cheese in addition to low-fat dairy or yogurt, on T2D risk in Britain, Europe, Japan and Australia (142–149). This was confirmed in a recent expert panel position paper (18), however, it remains important to note that the possibility of confounding is an aspect of observational studies which requires their review alongside well-designed and conducted RCTs in order to draw balanced consensus.

Overall, there is an extensive body of data from large observational trials of long duration, gathered over the last 20+ years, that support the association between total dairy intake and decreased incidence of CVD and/or T2D. Meta-analysis of prospective cohort studies of CVD-related incidence supports the recent characterization of this evidence as "convincing probable." Whilst it is not as yet clear as to dose-response patterns for dairy and CVD, there are strong associations that suggest different relationships with different dairy formats, specifically liquid milk, butter, cheese and yogurt. Similar observations are reported for T2D incidence. As noted above, confounding remains of concern if evidence is obtained solely from observational data. There is far less data available from human interventions trials from which consensus might be built on the relationships of CVD or T2D with total dairy or the dairy food groups milk, cheese, butter and yogurt. In particular there is an absence of dairy RCTs designed to evaluate endpoint CVD and T2D events, required in order to conclude that totality of evidence is convincing.

Randomized Controlled Trials (RCTs)

CVD

There is far less data available from RCTs from which consensus might be built on the relationships of CVD with total dairy or the dairy food groups milk, cheese, butter and yogurt (see **Table 1c**). Huth and Park (116) originally reported on short-term intervention studies and lipid biomarkers, where diets higher in SFA from whole milk and butter adversely increased LDL-C when substituted for carbohydrate or USFA. Adverse effects of high SFA vs high USFA (PUFA+MUFA) butter fat on lipid parameters of lean healthy men have previously been reported from our laboratory (150, 151). Meta-analysis (116) however also showed increased cardio-protective HDL-C, resulting in little or no worsening of the key CVD marker total cholesterol:HDL-C ratio. In addition cheese, when matched to the total and SFA content of butter, was shown to result in significant LDL-C lowering (116), confirming the outcomes from prior observational data. Other authors have since also questioned the role of dairy SFAs in CVD, with evidence that substitution of dairy SFA with (i) plant-derived n-6 PUFA vegetable oils (unless balanced with n-3 PUFA), and (ii) carbohydrate with high glycemic index (GI), may also worsen adverse cardiometabolic endpoints (117). Drouin-Chartier et al. more recently conducted an extensive umbrella review of data obtained from meta-analyses of RCTs, in addition to a large number individual RCTs. Whilst noting that these trials were conducted predominantly in healthy individuals, contrary to Huth and Park (116) they reported no adverse impact of high-SFA dairy consumption on multiple cardiometabolic variables, including various blood lipid fractions, blood pressure, inflammation and vascular function (126). The authors noted that their review narrative failed to address several potential confounders, including change in body weight, the multiple and varied control arms (both dairy and non-dairy dietary components) used in these dairy interventions, and the possibility of inadequate power in multiple trials where the CVD outcomes assessed were not primary end points. Nor did they evaluate potential publication bias. However, the conclusions drawn from this review, that there was no evidence from RCTs to support a detrimental effect of regular- or high-fat dairy on these many CVD risk factors, was in agreement with the observational trials discussed above.

Table 1c also summarizes meta-analyses of RCTs investigating effects of various specific dairy components on CVD risk factors. Brouwer et al. (75, 80) have reported adverse effects of *trans*-fats in large meta-analyses of up to 48 industrial CLA and 11 ruminant *trans*-fat RCTs. Various authors have reported adverse (96), positive (87), and mixed (89) effects of CLA on CV endpoints. Whilst positive effects have been reported in several meta-analyses of whey protein RCTs (30, 32).

A major gap in the interpretation of this evidence is the absence of large, long-term dairy RCTs where CVD incidence is the primary outcome. To date, the majority of data reported has been from short-term interventions in healthy individuals rather

TABLE 1c | Summary of meta analyses, systematic reviews and narrative reviews reporting CVD outcomes: RCTs.

References	No of studies	Study design	Dairy formats	Outcomes
Huth and Park (116)		Narrative review of RCTs and meta analyses	Total dairy, low-fat, high-fat, milk, cheese, butter, yogurt	Milk and butter increased plasma lipids (TC, LDL-C, HDL-C, apo B) in short term RCTs; however cheese, matched to butter for total-fat and SFA, significantly or borderline decreased LDL-C
Brouwer et al. (75)	39 studies: 29 industrial *trans* + 17 CLA + 6 ruminant fat RCTs	Meta analysis	*Trans*-fats vs. isoenergetic *cis*-monounsaturated fatty acids (MUFA, calculated)	Industrial *trans* fatty acids increased LDL-C:HDL-C ratio by 0.055 (95% CI 0.044, 0.066) vs. *cis* MUFA; by 0.038 (0.012, 0.065) vs. ruminant *trans* fatty acids, and 0.043 (0.012, 0.074) vs. CLA, for each energy % replaced
Brouwer et al. (80)	48 CLA + 11 ruminant fat RCTs	Meta-analysis	*Trans*-fats vs. *cis*-control fats	Ruminant *trans* fat and CLA both significantly increased LDL-C, LDL:HDL ratio, TC:HDL ratio vs. *cis*-control fats; CLA also decreased HDL-C vs. *cis*-control fats
Astrup et al. (117)		Narrative review	Yogurt, dairy	Yogurt and other dairy products decreased risk of CVD; supportive evidence but not conclusive.
Derakhshande-Rishehri et al. (95)	33 studies: 23 supplement + 10 food enriched RCTs	Systematic review and meta-analysis	CLA	Foods enriched with CLA or CLA supplements decreased LDL-C
Drouin Chartier, et al. (126)		Narrative review; umbrella review of meta analyses	Total dairy, high-fat, low-fat, milk, yogurt, cheese	Total or high-fat dairy, neutral effect (not adverse) on CVD lipid risk factors. Adverse effects of SFAs attenuated in complex food matrices of milk, cheese, yogurt
Wirunsawanya et al. (30)	9 RCTs	Systematic review and meta-analysis	Whey protein	Whey improved CVD risk factors in overweight and obese, but confounded by body weight and fat mass loss
Haghighatoost et al. (96)	11 RCTs	Systematic review and meta-analysis	CLA	CLA supplementation may worsen acute phase protein CRP and pro-inflammatory cytokine TNF-α
Li et al. (89)	3 RCTs	Narrative review	Uncommon dairy-origin fatty acids, including furan fatty acids, n-3 docosapentaenoic acid (DPA), and conjugated fatty acids	DPA has favorable effect on CV endpoints. Furan fatty acids and CLNA may be beneficial for CV health, but clinical evidence limited. CLA variable outcomes in risk factors RCTs, but no trials of CVD incidence.
Badeley et al. (32)	37 RCTs	Systematic review and meta-analysis	Whey protein	Whey significantly decreased SBP, DBP, waist circumference, HDL-C and TG; confounded by central fat mass (waist) loss

Apo B, apolipoprotein B; CI, confidence interval; CLA, conjugated linoleic acid; CLNA, conjugated linolenic acid; CRP, C-reactive protein; CVD, cardiovascular disease; DBP, diastolic blood pressure; DPA, docosapentaenoic acid; HR, hazard ratio; IHD, ischemic heart disease; HDL-C, high density lipoprotein –cholesterol; LDL-C, low density lipoprotein –cholesterol; MUFA, monounsaturated fatty acid; OCFA, odd chain fatty acids; RCT, randomized controlled trial; RR, relative risk; SBP, systolic blood pressure; SFA, saturated fatty acid; TC, total cholesterol; TG, triglyceride; TNF-α, tumor necrosis factor-α; T2D, type 2 diabetes.

than those as high risk, and has assessed accessible endpoints of CV risk. Only very few long-term interventions, such as the Finnish Dietary Prevention of Coronary Heart Disease studies for men and for women (152, 153), cluster-randomized 12 years intervention trials targeting decreased SFA through dairy replacement, have investigated CVD endpoints. These was conducted in two long-stay hospital wards where the majority of dairy fats were replaced by plant-origin n-6 PUFA fats for a 6 years duration, prior to crossover. Notably there was a significant decrease in serum cholesterol and incident CHD in men, smaller in women, when dairy was largely replaced by PUFA. The conundrum as to whether this decrease in clinical events was driven by decreasing SFA (dairy products replaced included whole milk and butter) or increasing PUFA (dairy substitutes of soybean oil in skimmed milk and high PUFA "soft" margarine) cannot be untangled. The critical nature of the comparator (or "control") arm in the interpretation of such trials has recently been highlighted in the wider discussion concerning the role of dietary SFA in prevention of CVD (154). In a systematic review of large dietary interventions, analyzing the data sets on the basis of SFA being replaced by PUFA, or CHO or by ignoring the composition of the substitute, led to quite different positive (PUFA) or null (CHO, ignore) relationships between SFA and CVD. This clearly highlights the need to conduct long duration RCTs trials where the non-dairy control arm is carefully planned and evaluated.

T2D

Again, as noted previously there is little data available from long-term RCTs that directly attribute amelioration of T2D risk factors, or prevention of T2D, through dairy intake. Rideout et al. conducted a small sample size, cross-over study of 2 x 6 months duration which reported improved insulin sensitivity (155), although notably this was low-fat dairy. A second cross-over trial also of 2 × 6 months duration conversely reported no effect of a high vs. low dairy intake on glycemic endpoints (156). A more recent study in hyperinsulinaemic adults that compared 6 weeks supplementation of adequate (three servings/week) vs. high (three servings/week) dairy intake however did not observe any difference in glucose or insulin outcomes including insulin

TABLE 1d | Summary of meta analyses, systematic reviews and narrative reviews reporting T2D outcomes: RCTs.

References	No of studies	Study design	Dairy formats	Outcomes
Turner et al. (158)	10 RCTs healthy and T2D risk, metabolic syndrome	Systematic review	Total dairy; no other lifestyle or other dietary change, including no weight change	4 studies had positive effect, 1 negative effect, and 5 no effect on homeostasis model assessment of insulin sensitivity (HOMA-IS)
Pasin and Comerford (27)	28 RCTs: 12 dairy foods + 16 dairy proteins; T2D and non-T2D	Meta-analysis	Milk, cheese, yogurt, whey protein, casein	Whey and casein improved glycemic endpoints (limited evidence ≤50 g/d protein). Cultured (yogurt and fermented drink "dough") and non-cultured (milk, cottage cheese) improved glycemic status in T2D; addition of probiotics further improved glycaemia, but limited evidence
Rahbar et al. (88)	32 RCTs healthy	Meta-analysis	CLA (*cis*9, *trans*11; *trans*10, *cis*12) supplements and enriched foods	CLA supplements (standardized mean difference, SMD, 0.075 mg/dL; 95% CI −0.099, 0.249) or CLA-enriched foods (SMD 0.126 mg/dL; 95% CI −0.100, 0.352) had no significant effect on FBG
Wirunsawanya et al. (30)	9 RCTs: 4 with glucose endpoints T2D risk ow/obese	Meta-analysis	Whey protein	Whey significantly decreased FBG; pooled mean difference (MD) 0.76; 95% CI 0.14, 1.38)
Mitri et al. (19)	15 RCTs T2D risk, ow/obese or metabolic syndrome	Narrative review	Total dairy vs. limited or no dairy; 8 studies weight maintenance	10 studies neutral effect, 5 studies improved glycemic endpoints including HbA_{1c}, FPG, insulin, HOMA-IR.
O'Connor et al. (159)	34 RCTs non-T2D	Systematic review and meta-analysis	Ruminant-origin dairy products elevated vs. low intake	Elevated dairy intake positively associated with FPG, [mean difference MD 0.07 mmol/L; 95% CI 0.01, 0.12], but not fasting insulin (MD −2.97 pmol/L; −7.05, 1.10) or HOMA-IR (SMD −0.07; −0.26, 0.12); negatively associated with HbA_{1c} in 4 studies (MD −0.09%; −0.09, −0.03). Quality of evidence low, high uncertainty
Sochol et al. (160)	30 RCTs	Systematic review and meta-analysis	Total dairy, milk, butter, cheese, yogurt	Diet with low-fat dairy decreases insulin resistance (HOMA-IR); mean difference (MD) −1.21 (95% CI−1.74, −0.67); may be confounded by decreased body weight and waist circumference
Badeley et al. (32)	37 RCTS ow/obese	Systematic review and meta-analysis	Whey protein isolate, concentrate, extract, supplement, powder, hydrolysate	Whey decreased FBS; weighted mean difference (WMD)−1.42, 95% CI 1.52, −1.31
den Hartigh (97)	13 RCTs, 5 with glucose endpoints, healthy ow/obese	Narrative review	CLA (*cis*9, *trans*11; *trans*10, *cis*12) supplements and dairy	CLA decreased body weight and body fat, but no glycemic improvement. May improve insulin resistance in high risk adults/children. In abdominally obese men, adverse increase in fasting glucose, insulin and CRP, and decreased insulin sensitivity.

CI, confidence interval; CLA, conjugated linoleic acid; FBG, fasting blood glucose; HOMA-IR, homeostatic model assessment-insulin resistance; HOMA-IS, homeostatic model assessment-insulin sensitivity; Mean difference, MD; RCT, randomized controlled trial; o/w, overweight; T2D, type 2 diabetes; SMD, standardized mean difference; WMD, weighted mean difference.

sensitivity, insulin secretion, and β-cell function (157). **Table 1d** presents a summary of systematic reviews, meta-analyses and narrative reviews. A systematic review of 10 RCTs of 1 week to 6 months duration, notably in weight stable individuals, failed to find conclusive evidence of improvement or detriment of dairy consumption on glycemic endpoints (158). Only 4 of 10 RCTs had a positive outcome on insulin sensitivity assessed as HOMA-IR. Pasin and Commerford reported on a meta-analysis of 28 dairy RCTs, with limited evidence of fermented dairy, probiotics and dairy proteins improving glycemic control (27). A more recent narrative review of 15 RCTs, of 2 weeks to 6 months duration, in individuals at risk of T2D concluded that whilst dairy does not have detrimental effects on glucose-related outcomes, positive outcomes are not universal (19). High dairy consumption was compared to a variety of no dairy and low dairy treatments. 10 of the studies showed no improvements with high dairy intake, whilst 5 studies showed improved HbA_{1c}, plasma glucose, and insulin resistance (HOMA-IR). O'Connor et al. have also recently published a review of 38 RCTs, in a heterogeneous meta-analysis of children (>5 years) and adults without T2D, where glycemic endpoints were measured in trials comprising a higher vs. lower dairy arm comparison (159). Notably elevated dairy intake was associated with worsening FPG, but not fasting insulin or HbA_{1c}, with the authors recording that the quality of evidence was low with high uncertainty. Sohol et al., have also recently conducted a meta-analysis of 30 RCTs (160) which reported that dairy, in particular low-fat dairy, decreased HOMA-IR assessed insulin resistance. The authors noted a series of limitations of their analysis including absence of discrimination between dairy food groups of milk, cheese, yogurt and components including dairy proteins, Ca and vitamin D, as well as missing data for HOMA-IR analyses in the original

papers and absence of baseline glycemic status. They also noted the significant heterogeneity in the RCTs included in the meta-analysis. **Table 1d** also summarizes a number of other reviews of RCTs where effects of the individual dairy components CLA and whey protein have been assessed. RCTs investigating effects of CLA (88, 97) have reported no significant positive effects on glycemic endpoints, whilst whey protein interventions (30, 32) have caused a significant decreased in FPG.

Again, a major omission in the interpretation of these RCTs is the fact that large, long-term interventions investigating effect of dairy on T2D incidence have not been conducted. To date, the majority of data reported has been from short-term interventions, of both healthy and impaired glucose tolerant individuals assessing commonly measured glycemic endpoints.

PROTECTIVE EFFECTS OF DAIRY FOOD MATRICES

Yogurt, Cheese

As referred to in the sections above, the complex matrix of dairy has in recent years been proposed to significantly modulate the nutritional properties of these food products (10, 25, 26, 161), acting both on acute and chronic endpoints. In 2017, a primarily Europe-based, expert panel concluded that evidence from RCTs supported the proposal that intake of whole dairy foods vs. single dairy constituents resulted in different cardiometabolic effects (25), and that this may underpin the protective inverse relationships commonly reported for fermented dairy products, yogurt and cheese. They proposed that food structures and processing methods may enhance interactions between nutrients in the dairy matrix, such that the nutritional value of the product could not be directly related to the simple nutrient content, and introducing the concept of "biofunctionality" of nutrients within dairy food structures. An example of a recent intervention study in healthy adults, where high-fat meals with matched total fat content were delivered across a range of dairy product formats, and where quite different postprandial response of serum lipid and lipoprotein fractions was observed (161).

Cheese provides a clear example, where the predicted adverse effects of SFAs on cardiometabolic health are ameliorated when these FAs are consumed as part of this complex high-fat matrix (10, 126, 129). These outcomes were unexpected in early studies where high-SFA dairy fat when given as cheese failed to raise total- and LDL-cholesterol compared with low-SFA control diets (162) or compared with butter fat (163). Evidence has grown in support of cheese being a neutral food group that fits into a healthy diet (164). This has recently been supported in an expert panel position paper where food matrix was reported to be a stronger determinant of health effects than SFA content *per se* (18). Cheese is manufactured in a wide range of formats which in turn themselves vary considerably in terms of food matrix, with corresponding variability in physiological response. For example, a recent acute study of commercial cheeses showed a soft format "cream" cheese to induce a greater increase in postprandial triglyceridaemia than butter, whilst hard format cheddar cheese did not. Purported to be linked to variance in GI disintegration processes (26). A longer-term RCT showed that dairy fat consumed within a cheese matrix lowered lipid markers vs. dairy fat delivered within alternative matrices (10).

In addition to cheese, there is evidence also that yogurt does not exert the adverse effects on blood lipids predicted solely by content of SFA (116, 117). In turn, the expert panel noted that consumption of yogurt is associated with decreased risk of T2D and cardiometabolic disease (18), as discussed previously in this review. Whilst a number of factors may contribute including low-total fat content, fermentation process and probiotic content, food matrix has been proposed as an additional significant contributor.

The mechanisms proposed to underpin these effects of food matrix are varied. Dairy products are highly varied themselves, with a vast array of both structure and processing methods used in production. Cheese is a good example. Based on macronutrient content cheese is a high-fat dairy product, as is butter, yet based on structural composition it is more similar to yogurt or liquid milk with a high protein, high calcium and high MFGM content (25). Decreased gastrointestinal (GI) absorption of lipid from dairy products rich in calcium, phosphorus and MFGM has been shown to directly increase lipid losses from the GI tract and so in turn decrease circulating lipid levels including chylomicron (diet-origin) TGs (165). Calcium within the dairy matrix has also been implicated in the suppression of LDL-cholesterol observed during chronic consumption of high fat cheese (25). Calcium phosphate has long been known to increase excretion of fecal bile acids, a mechanism by which LDL-cholesterol is suppressed since bile acids are synthesized from hepatic cholesterol and represent the primary pathway for cholesterol catabolism. It is less clear whether MFGM alone also affects fecal fat excretion, with mechanisms underpinning improvement in lipoprotein profile observed when, for example high-MFGM butter fat is consumed, being decreased GI cholesterol absorption and/or the regulation of expression of genes (25). Fermented dairy also has beneficial effects on blood lipids, hypothesized to be due to favorable effects on gut microbiota resulting in favorable SCFA profile. It has been confirmed that both yogurt and cheese increase levels of fecal SCFAs, with cheese intake increasing fecal butyrate, propionate, and malonate and decreasing fecal acetate and glycerol concentrations (25). Furthermore, there were significant correlations between fecal propionate and butyrate levels and LDL-cholesterol concentrations.

DISCUSSION

The contribution of dairy foods to cardiometabolic health has long been controversial. In particular, adverse effects that animal-origin dairy *trans* and SFAs may have on T2D and CVD outcomes. Consensus on the components of dairy in the diet has gradually been building, with some evidence of positive associations of whey protein and casein, milk fat globule membrane (MFGM) and polar phospholipids, vitamin D, and calcium with intermediary cardiometabolic endpoints. Conversely there is no consensus in support of the proposal that both *trans* and saturated dairy lipids have a consistent

TABLE 2 | Current dietary advice from both US and European medical associations for the prevention of CVD and T2D.

Medical Association	Year	Total fat	SFA	Dairy	Dietary advice
AHA Eckel et al. (170)	2013		5-6%, decrease SFAs including from dairy. Decrease *trans* fats	Include low-fat dairy	Adequate intake of plant foods including vegetable oils, nuts, whole grains, legumes, fish, poultry, and limited intake of sweets, sugar sweetened beverages and red meat.
ADA American-Diabetes-Association (171)	2019	20–35 en%	Should be limited, replace with USFA. No *trans* fats.	Include dairy—milk and yogurt	SFA replaced with USFA, and not refined CHOs. CHO intake should emphasize nutrient-dense high fiber, including vegetables, fruits, legumes, whole grains, as well as dairy products. No "one-size-fits-all" and no single ideal ratio of CHOs, fats, and proteins for T2D; meal plans should be individualized.
ESC Piepoli et al. (172)	2016	Focus on fat quality, not total fat	<10 en%, replace with USFA	Include low-fat dairy	Low in SFA replaced with PUFA; with focus on wholegrain products, vegetables, fruit, nuts and fish.
EASD Mann et al. (173)	2004	<35 en%	<10 en%	No dairy recommendations	Range of macronutrient ratios, comprising 45–60 en% CHO, 10–20 en% protein; include vegetables, legumes, fruits, wholegrains.
ADA/EASD joint consensus report (174, 175)	2018	20–35 en%	Should be limited, replace with USFA. No *trans* fats.	Include dairy—milk and yogurt	SFA replaced with USFA, and not refined CHOs. Medical nutrition therapy (MNT) comprising energy restriction and dietary quality. No single ideal ratio of CHO, fat or protein; CHO intake emphasizing nutrient-dense high fiber, including vegetables, legumes, fruits, whole grains, dairy

AHA, American Heart Association; ADA, American Diabetes Association; ESC, European Society of Cardiology; EASD, European Association for the Study of Diabetes; CHO, carbohydrate; T2D, type 2 diabetes; SFA, saturated fatty acids; USFA, unsaturated fatty acids; en%, percentage of total energy.

adverse effect on cardiometabolic health. Cohort studies report a neutral effect of *trans*-VA, but are not supported by RCT evidence that shows fatty acids containing *trans* double bonds to adversely alter serum cholesterol, irrespective of ruminant or industrial origin. In RCTs CLA both significantly improves and worsens CV risk factors but with little effect on glycaemia, and limited association with improved CV incidence reported in observational studies. For dairy SFA, evidence has been growing in support of no association or even a protective inverse relationship with cardiometabolic health, but largely driven by observational cohort studies. A clear research gap is the limited causal evidence of improvements in disease incidence obtained from intervention trials, with RCT evidence obtained predominantly from health individuals in trials of only short- or moderate-duration. In addition, it is proposed that the predicted adverse effects of these dairy lipids may be ameliorated when consumed within the complex food matrices of liquid milk, fermented cheese or yogurt.

In conducting this wide ranging review of the relationships between dairy and its myriad associated foods and components and cardiometabolic health, there are necessarily a number of limitations. Firstly this is a narrative and not systematic review, hence the ability to formally evaluate bias in its many forms in the large number of publications included both as individual studies or contributing to other narrative or systematic reviews is limited. The quality of the observational studies reviewed was highly variable, as were the design parameters of the RCTs. Study participants age, gender, anthropometric indices, health status and cardiometabolic risk factors also varied greatly between trials, both within and between within study type. It is not clear whether benefits are greatest in individuals identified as at risk of later disease. In RCTs sample size, composition of the comparator ("control") arm, baseline dairy intake, dairy product and comparator dose, and duration of intervention are examples of important parameters that are not adjusted for in a narrative review.

Currently, there is little consensus by International dietary guidelines on the recommended fat content of dairy, and whether continued focus on consumption of low-fat dairy is warranted. The issue is complex, with both the quality and quantity of lipids contributed to the diet through dairy needing to be considered, with potential for higher fat products to promote a high energy density diet (166, 167) and body weight gain (168). Dietary diversity, i.e., "eat a variety of foods" has become a gradually accepted recommendation to promote a healthy diet and reduce risk of cardiometabolic disease, however recent evidence from cohort studies has shown this greater diversity to be associated with poor eating patterns and in turn weight gain and obesity. For example, a higher intake of processed foods, refined grains and also sugar sweetened beverages (SSBs), and a lower intake of unprocessed foods such as fish, fruits, and vegetables (169).

Table 2 presents the current dietary advice from both US and European medical associations for the prevention of CVD and T2D, where low-fat dairy is most commonly included. The American Heart Association (AHA) diet and lifestyle recommendations (170, 176), amongst others, continue to promote a healthy eating pattern that emphasizes low-fat dairy

products alongside an adequate intake of plant foods including vegetable oils, nuts, whole grains, legumes, fish and poultry, and limited intake of sweets, SSBs and red meat. Conversely, the American Diabetes Association (ADA) annual guidelines continue to recommend a breadth of dairy products as part of a healthy diet, including milk and yogurt, for those with T2D (171), with no specific promotion of low-fat dairy. The European Society for Cardiology (ESC) guidelines (172) and the European Association for the Study of Diabetes (EASD) diabetes and nutrition study group (DNSG) (173) guidelines, expected to be updated in 2020 (177), both provide umbrella recommendations for SFAs to provide <10 en% and for low *trans*-fats. Alongside other healthy eating recommendations of consuming a diet high in vegetables, legumes, fruits and wholegrain cereals, EASD makes with no specific reference to dairy, whilst ESC recommends components of the Mediterranean Diet with minimal dairy for CVD prevention, and low-fat dairy for treatment of hypertension. A joint consensus report on management of hyperglycemia from ADA (174) and EASD (175) published concurrently in 2018 included diet and physical activity recommendations, with focus on medical nutrition therapy (MNT) of both energy restriction and optimal dietary quality including vegetables, legumes, fruits and whole grains, in addition to inclusion of dairy. This is part of a "no-one-size-fits-all" answer to diet and T2D by the ADA in their most recent 2019 Consensus Report (171).

The contribution of bovine milk and its myriad of products to cardiometabolic health is an area of active and still developing research. Large cohort studies, systematic reviews and meta-analyses now point to a neutral or even a protective inverse relationship of dairy with CVD and T2D, but long-term RCTs are as yet lacking. Such long-term well-controlled studies are required in order to build the cause and effect evidence base that is necessary in order for consensus in this long controversial area of nutritional science to finally be reached.

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The author confirms being the sole contributor of this work and has approved it for publication.

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Comparison of the Bifidogenic Effects of Goat and Cow Milk-Based Infant Formulas to Human Breast Milk in an *in vitro* Gut Model for 3-Month-Old Infants

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Human milk contains prebiotic components, such as human milk oligosaccharides (HMOs), which stimulate the growth of specific members of the infant gut microbiota (e.g., *Bifidobacteria*). Plant-based or synthetic oligosaccharides are often added to infant formulas to simulate the bifidogenic effect of HMOs. Cow milk, the most common source of protein in infant formula, and goat milk, used increasingly in the manufacture of infant formula, contain naturally-occurring prebiotics. This study compared the upper gastrointestinal digestion and subsequent colonic fermentation of human milk vs. goat and cow milk-based infant formulas (goat IF and cow IF, respectively), without additional oligosaccharides using an *in vitro* model for 3-month-old infants based on the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®). First, a dialysis approach using 3.5 kDa membranes was demonstrated to simulate small intestinal absorption of carbohydrates in conditions similar to those *in vivo*. During the *in vitro* digestion experiment, oligosaccharides were detected in human milk and goat IF but barely detected in the cow IF. Further, all three milk matrices decreased colonic pH by boosting acetate, lactate, and propionate production, which related to increased abundances of acetate/lactate-producing *Bifidobacteriaceae* for human milk (+25.7%) and especially goat IF (33.8%) and cow IF (37.7%). Only cow IF stimulated butyrate production which correlated with an increase in *Lachnospiraceae* and *Clostridiaceae*. Finally, *Enterobacteriaceae* and *Acidaminococcaceae* also increased with all three milk matrices, while production of proteolytic metabolites (branched-chain fatty acids) was only detected for the cow IF. Overall, goat and cow milk-based formulas without added oligosaccharides impacted gut microbial activity and composition similarly to human milk. This suggests that even without supplementation of formula with oligosaccharides, whole goat milk, whole cow milk and cow milk ingredients already supply compounds in formulas that exert beneficial bifidogenic effects. Further clinical research is warranted to elucidate the effect of whole goat milk-based formulas on the infant gut microbiome.

Keywords: human milk, infant formula, goat milk, cow milk, gut microbiota, short-chain fatty acid (SCFA)

INTRODUCTION

Microbial communities inhabit the human bowel and carry out diverse and complex biochemical processing of compounds that escape digestion and absorption along the upper gastrointestinal tract (GIT). The human gut microbial communities are established just after birth and strongly affected by subsequent dietary patterns such as breastfeeding or formula feeding and introduction of solid food (1–3). Prebiotics are defined as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species, already resident in the colon" (4). With respect to the infant diet, compounds with interesting prebiotic function are human milk oligosaccharides (HMOs). Due to their ability to resist acidic gastric conditions and enzymatic degradation in the upper GIT, they can exert their prebiotic effect in the lower GIT by specifically acting as nutrients and promoting the growth of *Bifidobacteria* and *Lactobacilli* in the colon (3, 5–7). *Bifidobacteria* are particularly well-adapted for the utilization of HMOs (8). A microbiota that is dominated by *Bifidobacteria* is considered protective, as it may activate the immune system and inhibit pathogens (9, 10). Some evidence suggests that *Bifidobacteria* and the production of short chain fatty acids (SCFAs) by gut bacteria may also protect against the development of allergy in infants (2, 3, 11).

Plant-based or synthetic oligosaccharides are often added to infant formulas to simulate the bifidogenic effect of HMOs. Infant formulas are commonly made from cow milk-derived ingredients (skim milk and whey protein powders), but goat milk is also a suitable milk source for formulas (12–14). Both goat milk and cow milk contain naturally-occurring oligosaccharides, albeit at lower concentrations and diversity than human milk (15, 16). However, compared to the profile of cow and sheep milk oligosaccharides, the profile of goat milk oligosaccharides is closer to that of human milk (15), with higher concentrations of fucosylated oligosaccharides and sialyloligosaccharides (16). Goat milk has also 4 and 10 times more oligosaccharides than cow milk and sheep milk, respectively (16). Goat milk has both acidic and neutral oligosaccharides, many of which are structurally comparable to HMOs (17–19). Therefore, the use of whole goat milk or specific goat milk fractions may provide some prebiotic benefits for the development of the maturing gut of the formula-fed infant.

Some studies have confirmed the potential of goat milk to influence the intestinal microbial community and metabolism in rodents (20, 21). Oligosaccharides isolated from goat milk promoted the growth of *Bifidobacteria* in *in vitro* models (22, 23). *Bifidobacteria* were also the most abundant microbes in stools of 2-month-old infants fed human milk, whole goat milk formula or whey-based cow milk formula (24). While there were no significant differences between the formula groups in the abundance of *Bifidobacteriaceae* or subspecies type, diversity analysis suggested that DNA sequences of microbiota were more similar when comparing the breast-fed and whole goat milk formula-fed infant groups than breast-fed and whey-based cow milk formula-fed infant groups (24). The aim of this study was to further investigate the observations made by Tannock et al. (24), by studying the effects of a whole goat milk-based and a whey-adjusted cow milk-based infant formula without any added prebiotics in comparison to human milk, using an *in vitro* model for 3-month-old babies, based on the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®).

TABLE 1 | Macronutrient composition of the goat (goat IF) and cow (cow IF) milk-based infant formulas.

	Units (per 100g powder)	Goat IF	Cow IF
Energy	kJ	2,200	2,200
	kcal	510	510
Protein	g	10.1	10.1
Whey	%	20	63
Fat	g	26.7	26.7
Milk fat	g	13.1	8.2
Saturated	g	10.4	9.8
Mono-unsaturated	g	12.1	10.3
Poly-unsaturated	g	4.2	6.6
Carbohydrate	g	57.4	57.4
Lactose	g	57.4	57.4

MATERIALS AND METHODS

Chemicals and Test Products

All chemicals were obtained from Sigma-Aldrich (Overijse, Belgium) unless stated otherwise. Human milk was from a mother in the third month of lactation. Informed consent was obtained prior to milk collection and local ethics approval was granted by the Ethics Committee of Ghent University Hospital (Belgian registration number B670201523541). Further, two infant formulas were tested, one made from whole goat milk (goat IF) and one made from cow milk protein ingredients (cow IF) (**Table 1**). Infant formulas were manufactured by Dairy Goat Co-operative (N.Z.) Ltd (Hamilton, New Zealand) from pasteurized whole milk (goat or cow milk), skim milk powder (cow IF only), whey protein powder (cow IF only), lactose, vegetable oils (high oleic sunflower oil, coconut oil, canola oil, sunflower oil, soybean oil), minerals, marine fish oil (source of DHA), microbial oil (source of arachidonic acid), and vitamins. Neither formula had added oligosaccharides. Formulas were prepared by mixing 6.6 g of powder to 100 mL with water. This is half the concentration used when preparing a bottle feed but was required for the lactose absorption simulation experiment as described later, and therefore human milk was also diluted 1:1 with water.

Experimental Design

The general reactor setup was adapted from the SHIME®, representing the GIT of the human adult as described by Molly

Abbreviations: BCFA, branched-chain fatty acids; GIT, gastrointestinal tract; HMO, human milk oligosaccharide; HPAEC-PAD, high-pressure anion-exchange chromatography with pulsed amperometric detection; IF infant formula; MFGM, milk fat globule membrane; SCFA, short-chain fatty acid; : SHIME, simulator of the human intestinal microbial ecosystem.

et al. (25), to the digestive conditions of 3-month-old infants. Similar as how van den Abbeele et al. (26) adapted model parameters for human adult simulations, operational parameters for infants were adapted from the international consensus method of the INFOGEST consortium (27) that was recently extended for young infants (28). All three test products (cow IF, goat IF and human milk) were first subjected to upper gastrointestinal digestive and absorptive processes, after which they underwent colonic incubations (**Figure 1**). The treatment effects were compared to a blank consisting of water. All experiments were conducted in triplicate.

Upper GIT Simulation of Milk Matrices

Except for the oral phase, which was omitted to simulate infant feeding condition, the residence times in the gastric and small intestinal incubations were similar to human adult conditions (26). Sixty milliliter of diluted milk matrices were mixed with simulated saliva medium (40 μL of solution containing 30 mg/mL α-amylase from porcine pancreas; 10% of the human adult level), pepsin (0.51 mL of a solution containing 20 mg of pepsin (R5015, Fiers, Zedelgem, Belgium; 18% of human adult level) per mL of 5 mM HCl), lipase (1.34 mL of a solution containing 50 mg/mL of Lipase from Rhizopus Oryzae; 100% of human adult level), lecithin (0.31 mL of a solution containing 13.5 mg/mL lecithin; 100% of human adult level), and gastric juice (2.3 mL of a solution containing 0.65 g KCl/L and 3.65 g NaCl/L). This mixture was incubated for 2 h at 37°C, during which a sigmoidal pH decrease was applied from 5.5 to 3.2 by adding 1M HCl. At the end of the incubation, the volume was increased to 75 mL with distilled water after which 10 mL was sampled. To the residual volume, 45 mL of a standardized enzyme and bile solution was added resulting in final amylase, lipase, chymotrypsin, bovine bile salts (BD Difco Oxgall, BD biosciences, Erembodegem, Belgium) and trypsin levels of 0%, 10, 50, 60, and 90% that of the human adult levels, respectively. Following a 30-min incubation at a pH of 6.5 and at 37°C, with stirring, a subsequent 3 h incubation at pH 6.7 was performed during which the intestinal content was incubated at the inside of a 3.5 kDa cut-off cellulose membrane, with the outside containing a dialysis solution (3.75 g/L $NaHCO_3$). The ratio of intestinal content:dialysis solution was 1:2 and the dialysis solution was refreshed hourly to ensure a high diffusion gradient and maintain a driving force for dialysis. At the end of the small intestinal incubation, qualitative oligosaccharide profiles were determined in the intestinal content via HPAEC-PAD.

Three types of membranes (0.1–0.5 kDa, 0.5–1.0 kDa, and 3.5 kDa) using three types of compounds [lactose, lactalbumin, and fructo-oligosaccharides (FOS)] were tested (results not shown). The 3.5 kDa membrane led to the highest absorption rate of lactalbumin and lactose. However, a small fraction of FOS was absorbed with the 3.5 kDa membrane but not with the other two membranes. Hence, the 3.5 kDa membrane was chosen as the best compromise to minimize absorption of non-digestible carbohydrates and maximize removal of lactose and proteins to best reflect *in vivo* conditions. During a pre-test, the dialysis via a 3.5 kDa cut-off membrane, as described previously by van den Abbeele et al. (26), was validated by using two reference molecules (lactose and FOS), while using same volumes and refreshment procedures as outlined above.

Oligosaccharide Profiling and Lactose Analysis via HPAEC-PAD

Concentrations of lactose during the pretest (to validate the dialysis membranes) were measured through high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), using a ICS-3000 chromatograph (Dionex, Sunnyvale, CA, USA) equipped with a CarboPacPA20 column (Dionex). The mobile phase, at a flow rate of 0.5 mL/min, consisted of ultrapure water (eluent A) and 100 mM NaOH (eluent B). The following gradient was applied: 0 min, 95% A and 5% B; 20 min, 80% A and 20% B (linear change); 25 min, 80% A and 20% B; 26 min, 0% A and 100% B (linear change); 29 min, 0% A and 100% B; 30 min, 95% A and 5% B (linear change) and 32 min, 95% A and 5% B. Sample preparation involved initial dilution of the sample with ultrapure water followed by deproteinization with acetonitrile (1:1), centrifugation (24,400 × g, 10 min) and filtration (0.2 μm PTFE, 13 mm syringe filter, VWR International) prior to injection (5 μL) into the column. Calibration was performed using external standards.

Analysis of absorption and degradation of FOS (reference compound used during pretest to optimize absorption simulation), goat IF, cow IF and human milk during passage through the upper GIT was performed with HPAEC-PAD using a ICS-3000 chromatograph (Dionex) equipped with a CarbopacPA200 column (Dionex). The mobile phase, at a flow rate of 0.5 mL/min, consisted of ultrapure water (eluent A), 100 mM NaOH (eluent B), and 100 mM NaOH and 1M CH3COONa. The following gradient was applied: 0 min, 90%, 10% B, and 0% C; 7 min, 90%A, 10% B, and 0% C; 9 min, 50% A, 50% B, and 0% C (linear change); 25 min, 50% A, 50% B, and 0% C; 28 min, 41.75%, 56% B, and 2.25% C (linear change); 38 min, 41.75%, 56% B, and 2.25% C; 51 min, 6% A, 82% B, and 12%C (linear change); 61 min, 6% A, 74% B, and 20%C (linear change); 76 min, 6% A, 74% B, and 20%C; 85 min, 0% A, 60% B, and 40% C (linear change); 90 min, 90% A, 10% B, and 0% C (linear change); 95 min, 90% A, 10% B, and 0% C. Sample preparation involved initial dilution of the sample with ultrapure water followed by deproteinization with acetonitrile (1:1), centrifugation (24,400 × g, 10 min) and filtration (0.2 μm PTFE, 13 mm syringe filter, VWR International) prior to injection (5 μL) into the column. Qualitative fingerprints were generated by plotting the elution time (in min) against the detected signal (in nC).

Colonic Incubation of Milk Matrices

After dialysis the intestinal content was subjected to a simulated colonic incubation as described by Marsaux et al. (29). Briefly, 49.5 mL colonic background medium [K_2HPO_4 4.8 g/L; KH_2PO_4 14.9 g/L; $NaHCO_3$ 2.0 g/L; yeast extract 2.0 g/L; peptone 2.0 g/L; mucin 1.0 g/L; cysteine 0.5 g/L; polyoxyethylene (20) sorbitan monooleate 2.0 mL/L] was added to reactors, already containing 20 mL of upper GIT suspension. The reactors were sealed with rubber stoppers and rendered anaerobic by flushing with N_2, after which 0.5 mL of a fecal inoculum was added. Stool

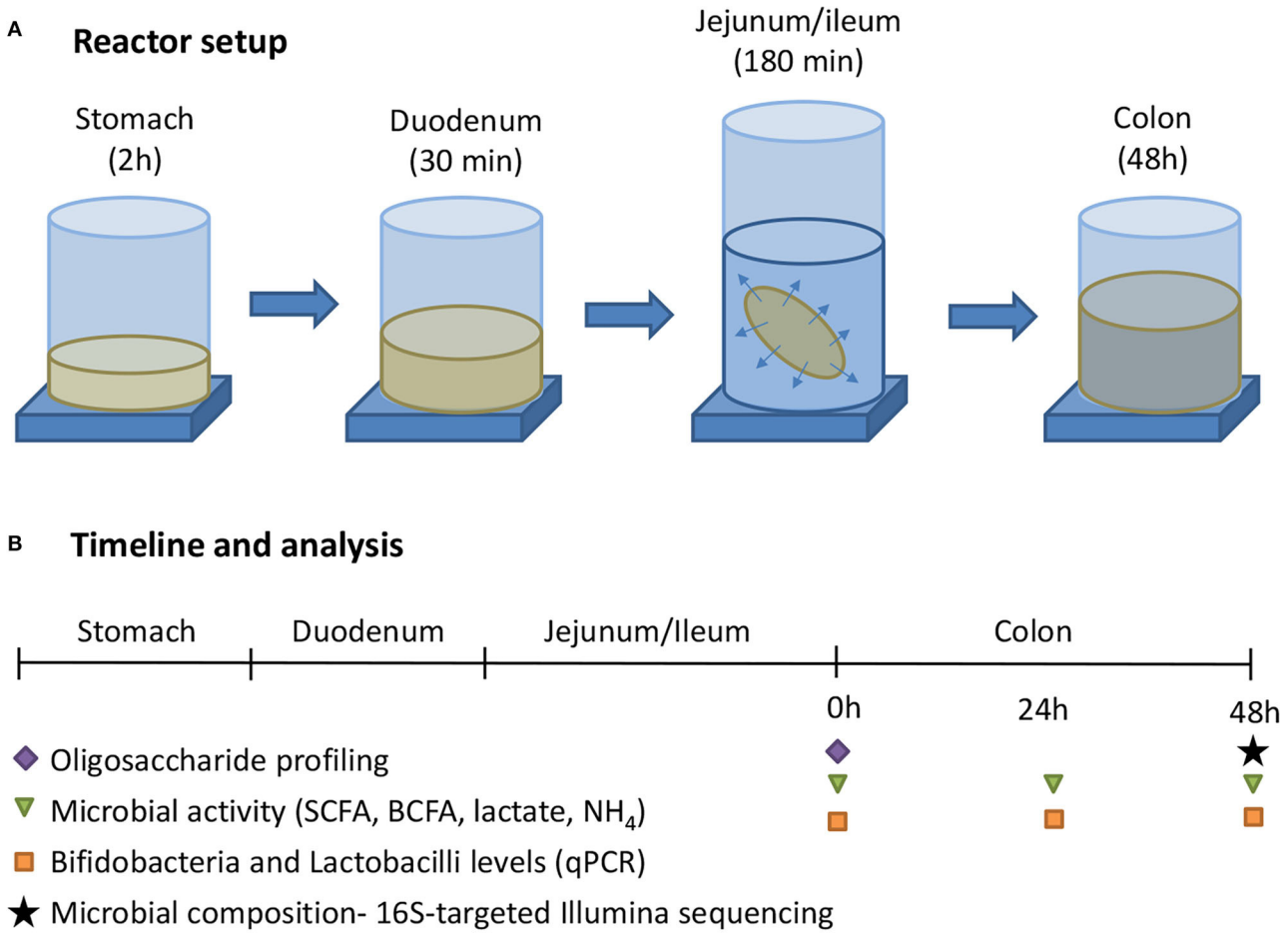

FIGURE 1 | (A) The experimental procedure consisted of a sequential incubation of the milk matrices (or blank = water) in the simulated stomach, duodenum, jejunum/ileum (with simulation of small intestinal absorption via static dialysis), and colon. **(B)** Along the experiment, samples were collected for analysis of the oligosaccharide fractions, together with microbial activity, and composition.

samples of 3-month-old infants were collected according to the ethics approval from the Ethics Committee of Ghent University Hospital (Belgian registration number B670201523541). The stool inoculum from three 3-month-old infants were pre-screened with galactooligosaccharide and FOS (ratio 9:1) to select a representative inoculum. The selected stool inoculum from one donor showed a microbial profile that was very similar to that of young infants (i.e., high *Bifidobacterium* levels and high production of acetate and lactate) whereas the stool inocula from the other two donors were more similar to that of adults. The inoculum was prepared by suspending a freshly collected fecal sample at 7.5% (w/v) in anaerobic phosphate buffer (K_2HPO_4 8.8 g/L; KH_2PO_4 6.8 g/L; sodium thioglycolate 0.1 g/L; sodium dithionite 0.015 g/L). Throughout the 48 h incubation (at 37°C; **Figure 1**), samples were collected at 0, 24, and 48 h for microbial metabolic activity analysis [pH, gas, SCFA, lactate, and branched-chain fatty acid (BCFA) production] and *Bifidobacterium* quantification (via qPCR). At the final time point (48 h), in-depth community analysis via 16S-targeted Illumina sequencing was performed.

Microbial Activity

Metabolic activity analysis at 0, 24, and 48 h included measurement of pH [measured via Senseline pH meter F410 (ProSense, Oosterhout, The Netherlands)] and gas production [measured via a pressure meter (hand-held pressure indicator CPH6200; Wika, Echt, The Netherlands)]. Further, total SCFA production was determined as the sum of acetate, propionate, butyrate and BCFAs (sum of isobutyrate, isovalerate, and isocaproate). The quantification method has previously been described by de Weirdt et al. (30). Ammonium determination was performed via a steam-distillation method followed by a titration as reported by de Boever et al. (31). Lactate was measured using a D-lactate/L-lactate kit (R-Biopharm, Mannheim, Germany).

Microbial Composition

Samples from the colonic incubation were collected at 0, 24, and 48 h for assessment of microbial composition. Abundance of *Lactobacillus* and *Bifidobacterium* were quantified via qPCR. DNA was isolated from 1 mL of samples as described before

by Boon et al. (32), with modifications as described by Duysburgh et al. (33). Subsequently, qPCR was performed on a QuantStudio 5 Real-Time PCR system (Applied Biosystems, Foster City, CA, United States). Each sample was run in technical triplicate and outliers with more than 1 CT difference were omitted. The qPCR for *Bifidobacteria* was performed as described previously by Rinttilä et al. (34), with the Bif243F (5′-TCGCGTCYGGTGTGAAAG-3′) and the Bif243R (5′-CCACATCCAGCRTCCAC-3′) primers, while *Lactobacilli* were quantified according to Furet et al. (35) with Llac05-F (5′-AGCAGTAGGGAATCTTCGGCA-3′) and Llac02-R (5′-GGGTAGTTACCGTCACTTGATGAG-3′) primers. Results are reported as log(16S rRNA gene copies/mL).

To get insight into the changes in relative abundance of the different microbial groups at overall community level, samples collected at 48 h were assessed via 16S-targeted Illumina sequencing (LGC genomics GmbH, Berlin, Germany) as described by Van den Abbeele et al. (36). Briefly, results obtained from the Illumina Miseq platform with v3 chemistry were presented as proportional values vs. the total amount of sequences within each sample and combined at family level. The reciprocal Simpson Diversity index was calculated as a measure of bacterial diversity, both in terms of species richness and evenness (37). The data used to create the OTU table that was used as a basis for the microbial community analysis in this paper have been deposited in the National Center for Biotechnology Information (NCBI) database (PRJNA675453).

Statistics

Incubations were run in triplicate. All data analyses were conducted using SAS 9.4. Data on production of gas, pH and ammonium, and *Bifidobacteria* abundance (log10 transformed) were analyzed using repeated measures ANOVA followed by Tukey *post-hoc* test. Lactate and SCFA data were analyzed using one-way ANOVA followed by Tukey *post-hoc* test. A $P \leq 0.05$ was considered to be significant. All values are reported as means ± standard deviation (SD).

RESULTS

Optimization of Simulation of Small Intestinal Absorption

First a novel dialysis approach was validated (using 3.5 kDa membranes) that would retain indigestible oligosaccharides at the inside of the membrane (simulated intestinal content), while allowing for diffusion (simulated absorption) of small mono- and disaccharides that are normally absorbed *in vivo* after digestion. FOS and lactose were used as reference substrates for the former and latter, respectively. First, upon comparison of chromatograms obtained via HPAEC-PAD for FOS (**Figures 2A,B**) vs. respective blanks (**Figures 2C,D**), it followed that the oligosaccharide fraction mainly eluted between 30 and 60 min, while the blank contained background peaks between 0 and 10 min. As only minor peaks appeared between 30 and 60 min in the chromatograms of samples of the dialysate upon applying the 3.5 kDa membrane (**Figure 2B**), the membrane was able to retain part of the oligosaccharide fractions of FOS. Further, dialysis with the 3.5 kDa membrane removed 52.2% of the lactose (data not shown). Therefore, the 3.5 kDa membrane was used for subsequent upper GIT simulations.

Small Intestinal Incubations With Milk Matrices

Figure 3 shows the HPAEC-PAD chromatograms of the different test products at the end of the small intestinal incubation. The oligosaccharides can be seen as peaks from 30 min retention time onwards. Following digestion, human milk contained the most oligosaccharides, followed by goat IF. Cow IF had relatively few oligosaccharides.

Colonic Incubations—Microbial Metabolic Activity

Gas production was used as a measure for microbial activity. The average (± SD) gas production (kPa) during the first 24 h was 81 ± 6 for goat IF, 75 ± 3 for cow IF, and 85 ± 1 for human milk. All test products resulted in increased gas production compared to the blank incubation (33.9 ± 0.6) ($P < 0.0001$). Human milk and goat IF resulted in more gas production compared to cow IF, but only statistically significantly for human milk ($P = 0.018$) in the first 24 h incubation period. Only a limited and highly variable amount of gas was produced in the second 24 h incubation (9 ± 5 for goat IF, 8 ± 1 for cow IF, 10 ± 3 for human milk, and 12 ± 0 for the blank incubation).

pH was recorded during colonic fermentation as a measure for the intensity of bacterial metabolism. At the beginning of the incubation period the pH of the medium was 6.6 to 6.8 for all test products and blank. After 24 h, pH decreased significantly ($P < 0.0001$) to 5.9 ± 0.0 for all test products, but only marginally (to pH 6.4 ± 0.0) for the blank incubation. There was no further change in pH during the second 24 h incubation period.

The production of SCFAs (acetate, propionate, and butyrate) was analyzed as markers for microbial carbohydrate metabolism. Lactate, produced by lactic acid bacteria in the gut, can also be rapidly converted to acetate, butyrate and propionate by lactate-utilizing gut bacteria (38). The change in concentrations of lactate and SCFAs from 0 to 24 h and 24 to 48 h is reported in **Table 2**. All test products resulted in increased lactate production during the 0–24 h interval as compared to the blank incubation. Highest lactate levels were observed upon fermentation of goat IF but it was not significantly different to the levels upon fermentation of the cow IF or human milk. The main contribution to total SCFAs was acetate, which increased during the first 24 h of incubation. During the second 24 h incubation period, concentrations of total SCFAs and acetate did not change. There were smaller increases in propionate concentrations in the first 24 h incubation. While propionate further increased in the second 24 h incubation, levels were <1 mM for all incubations. Butyrate concentrations increased <0.5 mM for all test products and the blank during both the 24 and 48 h incubation periods. However, highest butyrate levels were observed upon fermentation of cow IF in the first 24 h incubation period ($P < 0.0001$).

Microbial protein metabolism results in the production of BCFAs (isobutyrate, isovalerate, and isocaproate). BCFAs were

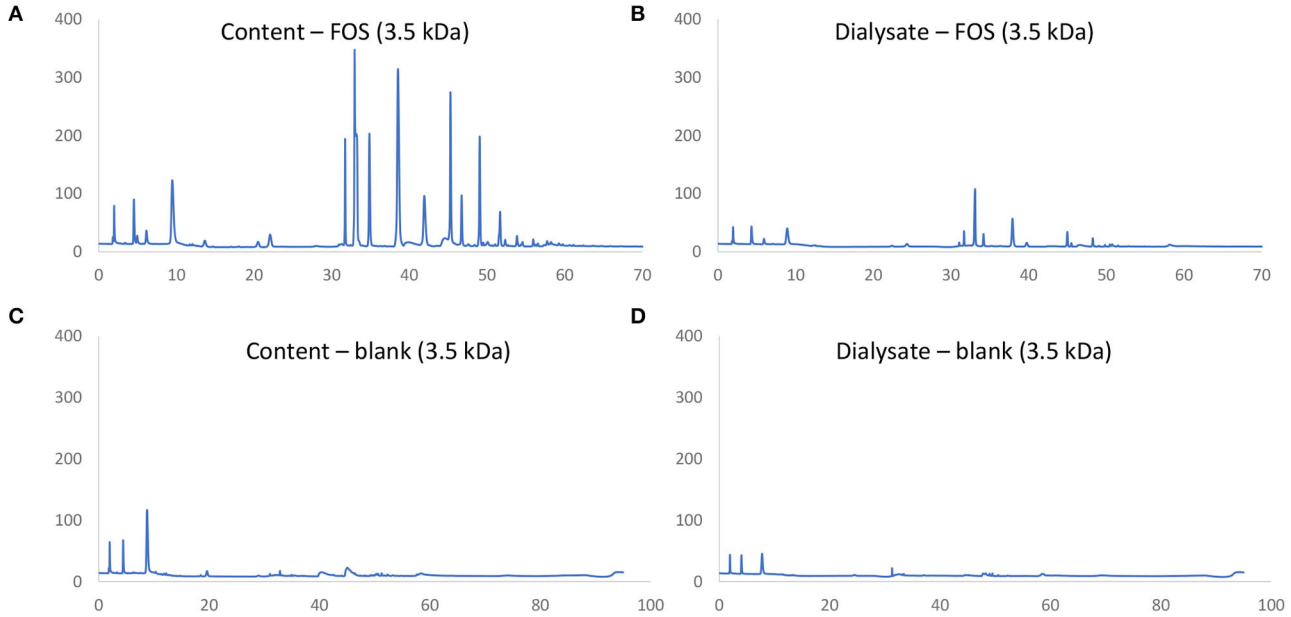

FIGURE 2 | Chromatographic fingerprint of signal (nC) in function of time (min) obtained through HPAEC-PAD analysis of samples in the simulated intestinal content **(A,C)** or absorbed fraction **(B,D)** upon upper GIT transit of FOS **(A,B)** or a blank **(C,D)** using specific 3.5 kDa dialysis membranes to simulate small intestinal absorption.

just above the detection limit in the blank incubation (0.03 ± 0.03 mM) during the first 24 h of incubation, but not in any of the test products. In the second 24 h, concentrations of branched SCFAs were 0.07 ± 0.04 mM from the incubations with blank and the cow IF. Both goat IF and human milk had no detectable change in BCFAs.

The production of ammonium (NH_4^+) that results from proteolytic activity of the gut microbiota was significantly reduced ($P = 0.0316$) by all products compared to the blank incubation. The highest ammonium production occurred during the 0–24 h time interval (59 ± 5 mg/L for goat IF, 50 ± 8 mg/L for cow IF, 57 ± 4 mg/L for human milk, and 87 ± 2 mg/L for the blank). During the second 24 h incubation period, ammonium concentrations were 24 ± 1 mg/L for goat IF, 28 ± 2 mg/L for cow IF, 35 ± 22 mg/L for human milk, and 39 ± 6 mg/L for the blank.

Colonic Incubations—Microbial Composition

The original donor sample selected for the *in vitro* dynamic digestive model was dominated by the *Bifidobacteriaceae* family, and in particular *Bifidobacteriaceae* operational taxonomic unit (OTU) (related to *Bifidobacterium Breve*). All test products resulted in a further increase in numbers of *Bifidobacteriaceae* as compared to the blank incubation ($P = 0.0001$), with the main increase being observed during the first 24 h of incubation (**Figure 4**). No differences were observed amongst the different test products.

At the start of the incubation, the bacterial inoculum was mainly dominated by bacterial species of the *Bifidobacteriaceae* (**Table 3**). *Lachnospiraceae* was the next most abundant bacterial species. *Lactobacillus* was below the limit of detection. Reciprocal Simpson Diversity index indices for goat IF, cow IF and human milk were 2.9, 2.8, and 2.9, respectively, compared to the blank incubation with an index of 6.4. These reflect a strong increase in abundance of *Bifidobacteriaceae* and *Enterobacteriaceae*, following the incubation with the test products (**Table 3**).

DISCUSSION

Human, goat and cow milk contain natural prebiotics such as oligosaccharides, lactose, nucleotides, and glycosylated proteins and lipids, albeit at different concentrations and diversity (15). HMOs are the third most abundant components of human milk and therefore have a greater prebiotic effect than other components (6).

All test products stimulated overall microbial activity as observed by a stronger pH decrease, increased gas production and production of health-related metabolites such as SCFAs and lactate as compared to the blank incubation. Gas production reflects microbial substrate fermentation while the decrease in pH was likely due to increased lactate and SCFAs. These observations are consistent with the effects of human milk on microbial activity in humans (3, 8) and studies on goat and cow milk in rodents (20, 21).

With respect to product-specific findings, it was found that human milk and goat IF digestion and fermentation resulted in a significantly greater gas production compared to cow IF, while human milk digestion, and fermentation resulted in a slightly greater increase in acetate and propionate production compared to either of the formulas. While both formulas resulted in

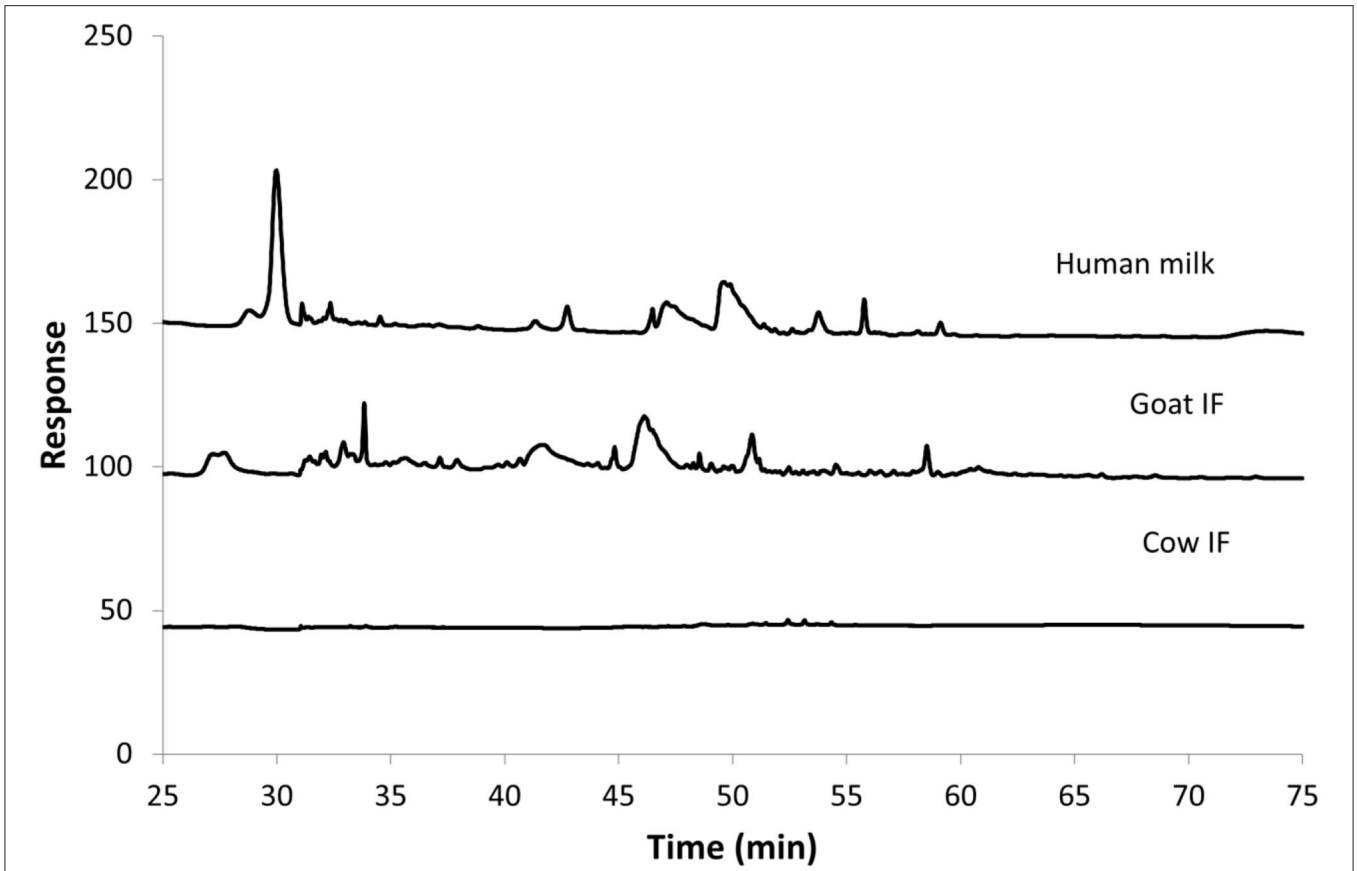

FIGURE 3 | Chromatogram from HPAEC-PAD analysis of Cow IF, Goat IF, and human milk at the end of small intestinal incubation. The oligosaccharide fraction is mainly present from retention time of 30 min.

TABLE 2 | Change in concentrations of lactate and SCFAs in the 0–24 h and 24–48 h period of colonic fermentation of the goat infant formula (goat IF), cow infant formula (cow IF), and human milk as compared to the blank control.

Concentration (mM)	Goat IF		Cow IF		Human milk		Blank	
	0–24 h	24–48 h	0–24 h	24–48 h	0–24 h	24–48 h	0–24 h	24–48 h
Lactate	8.87 ± 1.56^{a}	0.44 ± 0.25^{a}	7.70 ± 0.54^{a}	0.56 ± 0.26^{a}	8.26 ± 0.44^{a}	−0.97 ± 0.59^{b}	1.00 ± 0.06^{b}	−0.21 ± 0.04^{a}
Acetate	25.22 ± 2.21^{a}	−0.04 ± 1.53^{a}	24.61 ± 2.73^{a}	−0.55 ± 2.81^{a}	26.98 ± 1.00^{a}	0.44 ± 2.40^{a}	12.39 ± 0.53^{b}	2.49 ± 0.40^{a}
Propionate	3.64 ± 0.09^{b}	0.71 ± 0.11^{a}	3.57 ± 0.28^{b}	0.66 ± 0.35^{a}	4.27 ± 0.32^{a}	0.64 ± 0.25^{a}	2.53 ± 0.10^{c}	0.84 ± 0.11^{a}
Butyrate	0.12 ± 0.04^{c}	0.10 ± 0.11^{a}	0.32 ± 0.05^{a}	0.28 ± 0.14^{a}	0.00 ± 0.00^{d}	0.06 ± 0.06^{a}	0.22 ± 0.03^{b}	0.15 ± 0.04^{a}
Total SCFAs	28.99 ± 2.22^{a}	0.87 ± 1.75^{a}	29.28 ± 2.24^{a}	0.20 ± 2.54^{a}	31.25 ± 1.12^{a}	1.16 ± 2.64^{a}	15.16 ± 0.61^{b}	3.55 ± 0.55^{a}

SCFAs, short-chain fatty acids. Values for each metabolite with the same letter are not statistically different.

increased lactate production compared to the blank incubation, highest lactate levels were observed upon fermentation of goat IF. Lactate is an important metabolite in the human colon environment because it decreases the gut pH and acts as an antimicrobial agent (39), but also because it is the driver of a series of trophic interactions with other bacteria, resulting in the production of butyric and propionic acids (40). Thus, changes in lactate could have resulted either from a change in production or utilization. However, concentrations of propionate were similar between the two formulas and while butyrate was higher with cow IF, butyrate represented only 1–2% of the total output of SCFAs.

With regards to bacterial composition, all test products increased *Bifidobacteriaceae* and reduced species richness compared to the blank during the incubation period. The major change in bacterial composition was increased abundance of *Enterobacteriaceae*, mainly attributed to *Enterobacteriaceae* OTU (related to *Escherichia coli*). These changes are consistent with the production of SCFAs and lactate observed upon treatment with all test products. For example, *Escherichia coli* utilizes a wide

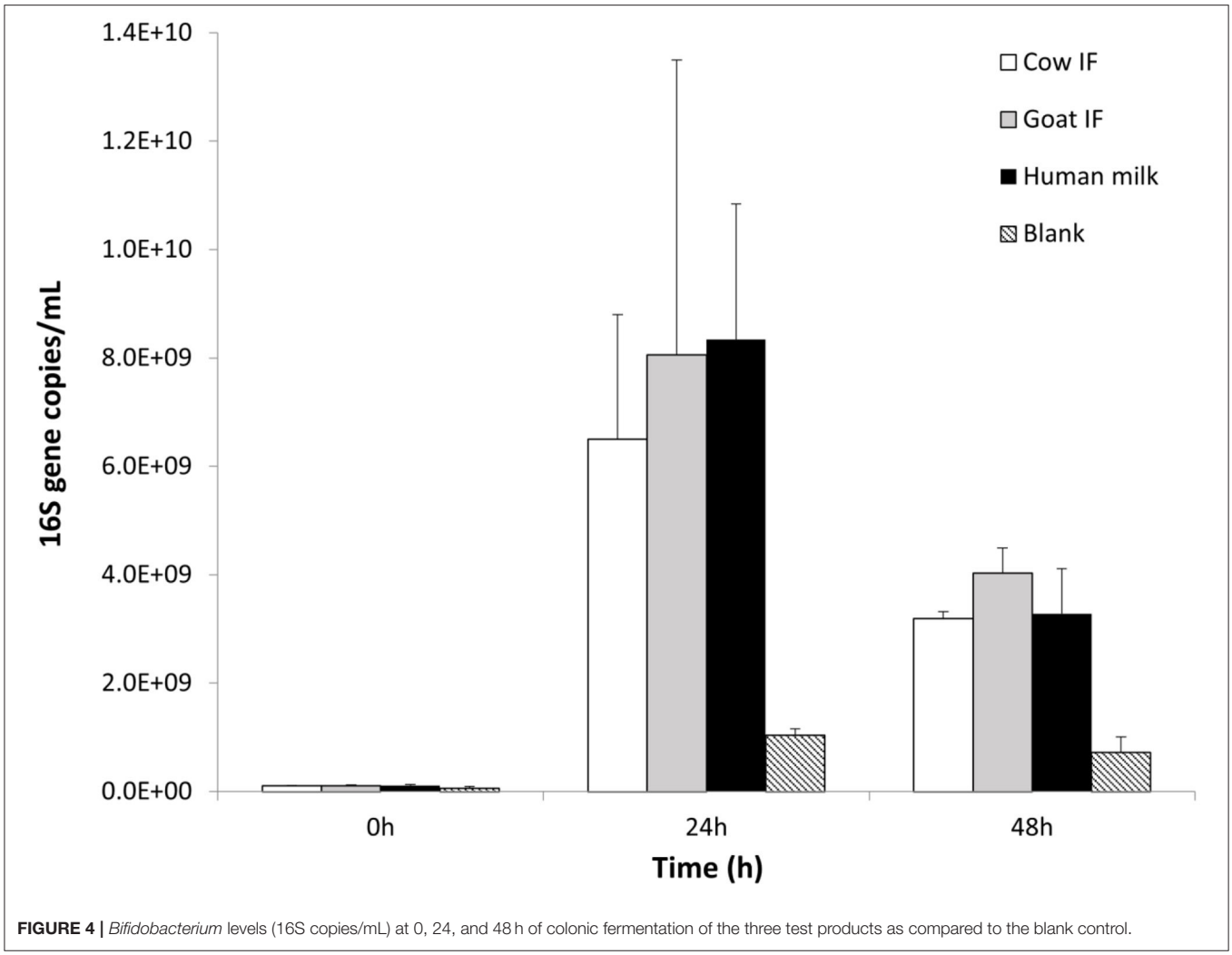

FIGURE 4 | *Bifidobacterium* levels (16S copies/mL) at 0, 24, and 48 h of colonic fermentation of the three test products as compared to the blank control.

TABLE 3 | Average ($n = 3$) abundance (%) of dominant bacterial families in the original inoculum and following 48 h incubation with digestive products of goat infant formula (goat IF), cow infant formula (cow IF), and human milk and with the blank.

Phylum	Family	Inoculum	Goat IF	Cow IF	Human milk	Blank
Actinobacteria	*Bifidobacteriaceae*	55.3	44.8	48.7	36.7	11.0
	Eggerthellaceae	0.5	0	0	0	2.0
Bacteroidetes	*Bacteroidaceae*	9.5	7.5	7.0	7.1	24.6
	Rikenellaceae	0.1	0	0	0	0
Firmicutes	*Acidaminococcaceae*	2.8	4.8	3.6	6.3	1.5
	Clostridiaceae	0.2	0.1	0.3	0.1	0
	Enterococcaceae	1.1	1.7	1.7	1.3	22.8
	Erysipelotrichaceae	5.0	0	0	0	0
	Lachnospiraceae	18.4	2.7	3.5	1.9	20.0
	Ruminococcaceae	1.0	0	0	0	0.4
	Streptococcaceae	0.3	0	0	0	0
Proteobacteria	*Burkholderiaceae*	0.5	0	0	0	0.1
	Enterobacteriaceae	5.3	38.4	34.9	46.5	17.4

variety of substrates including HMOs (41, 42) to produce acetate. Both *Bifidobacteriaceae* and *Enterobacteriaceae* are capable of producing high concentrations of lactate. *Acidaminococcaceae* also increased, mainly due to *Acidaminococcaceae* OTU (related to *Phascolarctobacterium faecium*), which is a known producer of propionate. While the abundance of *Bifidobacteriaceae* and *Enterobacteriaceae*, it is likely that levels of some bacterial species remained unchanged, which was not possible to demonstrate using proportional 16S sequencing but could be in the future using quantitative sequencing (43).

Lactate-producing bacteria are primary colonizers of the infant gut (44). Therefore, to prevent toxic accumulation, lactate must be used as a substrate by lactate-utilizing bacteria. This may result in H_2 production and accumulation, which may contribute to infantile colic symptoms such as acute bloating and cramping (44). A small study (44) showed that an imbalance between H_2-producing and H_2-utilizing bacteria was associated with infantile colic. In the present study, fermentation of goat IF resulted in higher lactate levels, albeit not significantly, and gas production was highest during fermentation of goat IF and human milk. It would be of interest to explore these results in future clinical settings.

In the present system, *Lactobacilli* levels remained below the detection limit, consistent with low levels during infancy reported in other studies (3, 24). After *Bifidobacteriaceae, Lachnospiraceae* was the next most abundant bacterial species in the inoculum. Tannock et al. (24) observed that when *Bifidobacteriaceae* abundance in stools of infants was high, *Lachnospiraceae* abundances tended to be low, suggesting there is a metabolically competitive interaction between *Bifidobacteriaceae* and *Lachnospiraceae*. In the present study, the percentage of *Lachnospiraceae* dropped after incubation with digestive products of formulas and human milk, suggesting that the digestive products from human milk and formula were more conducive to growth of *Bifidobacteriaceae* than *Lachnospiraceae*. Thus, similar to Tannock et al. (24), the present study shows that *Bifidobacteria* are maintained even in the presence of low levels of oligosaccharides in formula made with ruminant milks. In the present study, both formulas contained milk fat, but as the goat IF was made with whole goat milk without added whey, it contained higher levels of milk fat than the cow IF (50 vs. 30% of total lipids). As a result, the goat IF would be expected to contain more components of the milk fat globule membrane (MFGM). It is possible that glycoproteins and glycolipids associated with the MFGM may also act as growth substrates for *Bifidobacteria* and other bacteria (45–47). To note as well, that the cow IF in the present study contained more milk fat than standard infant formulas manufactured with skim milk and whey protein ingredients, which may have contributed to some effects on microbial activity and composition (46). In addition, both formulas contained DHA, which may play a role in the development of the microbiota and allergy in infants (48).

One of the limitations of the *in vitro* dynamic digestive model is that it lacks the full complement of the digestive system. For example, brush border enzymes, such as lactase, are not present and as a result there was no breakdown of lactose into galactose or glucose. All test products were diluted by half and then a 3.5 kDa dialysis membrane was used during the jejunal and ileal incubation phase to simulate the absorptive processes and to reduce undigested lactose passing into the colonic digestion phase. Dialysis reduced lactose concentrations by half what was present in the diluted test products. HPAEC-PAD analysis confirmed there was no loss of oligosaccharides after dialysis, which is important when performing mechanistic research on the effect of the test products on the colonic microbiota. The highest amount of non-digestible oligosaccharides at the end of the small intestinal incubation was present in human milk as compared to the goat or cow IF, consistent with much higher concentrations of oligosaccharides from human milk compared to ruminant milks (19). Even with the dialysis, it is possible that concentrations of lactose and amino-nitrogen presented to the bacteria within the inoculum may be higher than levels *in vivo*. However, levels of BCFAs that are indicative of protein fermentation (49) were only just detectable in the second 24 h of incubation with blank or cow IF when it may be expected that supply of fermentable carbohydrates becomes limiting. Thus, it is likely that protein digestion products at least had little impact on the outcomes of this study. While infants are able to digest large quantities of lactose, it can be expected that some lactose escapes digestion and absorption and is fermented by gut bacteria (50, 51).

The strength of the *in vitro* dynamic digestive model is the tight control of environmental factors influencing the microbiota. Thus, it is possible to be very confident that the stimulation of specific bacterial species is directly attributed to the inherent prebiotic properties of the products and not to other events. This, combined with the ability to test products in triplicate with the single inoculum, provides much greater reproducibility than using *in vivo* studies with infants.

CONCLUSIONS

All three milks stimulated microbial activity and increased *Bifidobacteria*, which are regarded as beneficial saccharolytic bacteria in infancy (3). Similar to Tannock et al. (24), both formulas impacted the gut microbial activity and community composition comparable to human milk, despite the relative absence of oligosaccharides in the formulas. This may be explained by the presence of naturally-occurring oligosaccharides, milk fat and MFGM within the formulas, in particular the formula made from whole goat milk, used in the present study. Further clinical research is warranted on the role of goat milk fat in formulas on the development of the gut microbiota in early life.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: NCBI; accession no. PRJNA675453.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Ghent University

Hospital. Written informed consent to participate in this study was provided by the participant or the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

CP developed the study concept. PV carried out the experiment and collected the data. SG, CP, and PV evaluated the data, wrote the manuscript, and proofread and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: SG and CP are employees of Dairy Goat Co-operative (NZ) Ltd, that financially supported the study and manufactured the formulas for use in this study.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The Milk Metabolome of Non-secretor and Lewis Negative Mothers

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Introduction: The functional role of milk for the developing neonate is an area of great interest, and a significant amount of research has been done. However, a lot of work remains to fully understand the complexities of milk, and the variations imposed through genetics. It has previously been shown that both secretor (Se) and Lewis blood type (Le) status impacts the human milk oligosaccharide (HMO) content of human milk. While some studies have compared the non-HMO milk metabolome of Se+ and Se− women, none have reported on the non-HMO milk metabolome of Se− and Le– mothers.

Method and Results: To determine the differences in the non-HMO milk metabolome between Se–Le– mothers and other HMO phenotypes (Se+Le+, Se+Le–, and Se–Le+), 10 milk samples from 10 lactating mothers were analyzed using nuclear magnetic resonance (NMR) spectroscopy. Se or Le HMO phenotypes were assigned based on the presence and absence of 6 HMOs generated by the Se and Le genes. After classification, 58 milk metabolites were compared among the HMO phenotypes. Principal component analysis (PCA) identified clear separation between Se–Le– milk and the other milks. Fold change analysis demonstrated that the Se–Le– milk had major differences in free fatty acids, free amino acids, and metabolites related to energy metabolism.

Conclusion: The results of this brief research report suggest that the milk metabolome of mothers with the Se–Le– phenotype differs in its non-HMO metabolite composition from mothers with other HMO phenotypes.

Keywords: human milk, metabolome, Lewis negative, oligosaccharide, energy metabolism, South Africa, non-secretor

INTRODUCTION

Human milk is the gold standard for infant nutrition as it provides essential nutrients for infant growth, as well as bioactive components such as human milk oligosaccharides (HMOs). While the variation of HMOs among different maternal HMO phenotypes has been widely studied (1–4), the impact of the maternal HMO phenotypes on other low-molecular-weight milk metabolites remains unclear. Metabolites other than oligosaccharides are thought to play important roles in infant health. For example, milk glutamate has been shown to impact appetite and growth (5), biogenic amines have been reported to provide protection against infectious disease (6), taurine has been recognized to contribute to neonatal brain development (7), and creatine appears to be essential for normal neural development (8). An understanding of how these metabolites change with HMO phenotype may be important to further understanding of the function of these metabolites in milk.

Maternal HMO phenotypes are determined by the activity of two genes: the secretor (Se) gene *fut2*, coding for α-1,2-fucosyltransferase 2 (FUT2), and the Lewis (Le) gene *fut3*, coding for α-1,3/1,4-fucosyltransferase (FUT3). FUT2 and FUT3 are responsible for the fucosylation of milk oligosaccharides. There are five monosaccharides upon which all HMOs are built: D-glucose, D-galactose, N-acetylglucosamine (GlcNAc), L-fucose and sialic acid (Neu5Ac) (9). At the core of the HMO structure is lactose, which can be sialylated to form α2-3 (e.g., 3′sialyllactose, 3'SL) or α2-6 (e.g., 6′sialyllactose, 6'SL) linkages to sialic acid, or fucosylated to form α1-2 (e.g., 2′FL), or α1-3 (e.g., 3FL) linkages to fucose. To form more complex HMOs, lactose can be elongated through a β1-3 linkage to lacto-N-biose (type I) or a β1-6 linkage to N-acetyllactosamine (type II). Lactose or the formed polylactosamine backbone can then be sialylated and/or fucosylated to create an additional 200 different oligosaccharide structures (10). FUT2 synthesizes 2'FL or lacto-N-fucopentose I (LNFP I) by attaching a fucose to lactose or lacto-N-tetraose (LNT), respectively. FUT3 synthesizes lacto-N-difucohexaose I (LDFH I) and lactodifucotetraose (LDFT) from LNFP I and 2'FL, respectively, by attaching an additional fucose. FUT3 can also directly transfer fucose to LNT, lactose, and lacto-N-neotetraose (LNnT) to form lacto-N-neotetraose II (LNFP II), 3FL, and lacto-N-neotetraose III (LNFP III), respectively (4). Additionally, the α-1,3-fucosyltransferases encoded by *fut4, 5, 6, 7,* and/or *9*, which are Se− and Le− independent, also play roles in attaching fucose to lactose, and thus 3FL and LNFP III can sometimes be observed in milk from Lewis negative (Le−) women (11, 12). It has been speculated that FUT1 α-1,2-fucosyltransferase 1 also participates in HMO fucosylation, as α-1-2-fucosylated HMOs have been observed in milk from Se− women (13). In human milk from Se+/Le+ women, 35–50% of the HMOs are fucosylated, 12–14% are sialylated, and 42–55% are non-fucosylated neutral (14).

While the Se and Le genes are important to generate a variety of HMOs in both free and conjugated forms, many individuals have polymorphisms in one or both of these genes making them non-functional. In European and American populations, the Le− frequency is between 4 and 6%, and 20% of the population are Se–, making Se–Le– extremely rare. In contrast, in certain African populations, over 30% of the population are Le− and ∼38% are Se− (15–17), which makes the probability of having Se–Le– mothers higher. The importance of functional Se and Le genes in infant development is an area of active research. One study showed that maternal secretor status appeared to be important for preventing diarrhea, as although the gut microbiota measured through 16S rRNA sequencing did not differ between infants of Se+ and Se− mothers, the prevalence of diarrhea was higher among infants of Se− mothers (18). Moreover, when these infants were provided iron supplements, infants of Se− mothers were more likely to experience a decrease in the abundance of *Bifidobacterium* and an increase in pathogens compared to infants of Se+ mothers (18). However, supplementation with galactooligosaccharides appeared to ameliorate the impact of iron supplementation (18).

Studies comparing the non-HMO milk metabolome from mothers who were phenotypically Se+ to Se− demonstrated no differences between groups (19, 20). We have previously reported on the milk metabolome at day 90 (21) and over the first month of lactation (22) in Se+Le+ and Se–Le+ women. We observed no significant difference in non-HMO metabolites between the two groups. To date, no studies have compared the non-HMO metabolome of milk from phenotypically Se–Le– mothers to any other phenotype. We hypothesized that the non-HMO milk metabolome from Se–Le– women would be similar to the other phenotypes 1 month after delivery. This brief research report provides preliminary data on the comparison of the milk metabolome between women with the Se–Le– phenotype and other phenotypes.

Abbreviations: HMOs, Human milk oligosaccharides; Se, secretor; Le, Lewis; FUT1, α-1,2-fucosyltransferase 1; FUT2, α-1,2-fucosyltransferase 2; FUT3, α-1,3/1,4-fucosyltransferase; GlcNAc, N-acetylglucosamine; Neu5Ac, sialic acid; LNFP I, lacto-N-fucopentose I; LNFP II, lacto-N-fucopentose II; LNFP III, lacto-N-fucopentose III; LNT, lacto-N-tetraose; LNnT, lacto-N-neotetraose; LDFH I, lacto-N-difucohexaose I; LDFH II, lacto-N-difucohesaose II; LDFT, lactodifucotetraose; 2'FL, 2'fucosyllactose; 3FL, 3-fucosyllactose; 3'SL, 3'sialyllactose; 6'SL, 6'sialyllactose; PCA, principal component analysis.

METHODS

Milk Sample Preparation

In this pilot study, to maximize the homogeneity of subjects (23), human milk samples were collected 1 month postpartum from 10 randomly-selected healthy women (age 29.8 ± 4.8, pre-pregnancy BMI 25.0 ± 2.9) in Cape Town, South Africa, who gave birth to term infants (50% male) through vaginal delivery, and practiced exclusive breastfeeding prior to sample collection. The exclusion criteria included antibiotic or probiotic treatment during the last trimester of pregnancy, and the breastfeeding period. Ethical approval for this study was provided by the University of Cape Town's Human Research Ethical Committee (HREC REF: 306/2014). Mature milk samples from mothers were collected after obtaining their consent. Women were asked to wash their hands, their nipple, and surrounding breast area with soap, then soak the breast area with chlorhexidine to reduce contamination by skin microbes, followed by washing with sterile water. A small volume of

milk was collected manually or with an electric breast pump into a sterile collection bottle after discarding the first few drops. Time since last feed was not recorded. After collection, samples were transported on ice and stored at −20°C until further processing. This study is a subset of a larger study on the relationship of milk short chain fatty acids and atopy (24).

Milk samples were prepared as previously described (25). Briefly, samples were thawed on ice, mixed, then 500 μL of each sample was filtered through Amicon Ultra 0.5 mL 3-kDa cutoff spin filters (Millipore Sigma, Burlington, MA, USA) at 10,000 × g for 15 min at 4°C to remove lipids and protein, as the study was interested in low-molecular-weight polar metabolites. Three hundred and fifty microliter of filtrate was mixed with 70 μL of deuterium oxide and 60 μL of standard buffer solution [consisting of 585 mM NaHPO4 (pH 7.0), 11.667 mM disodium-2,2-dimethyl-2-silapentane-5-sulfonate (DSS, internal standard), and 0.47% NaN_3 in H_2O] in a 1.5 mL Eppendorf tube (25). Four hundred and sixty microliter of the mixture was transferred to a nuclear magnetic resonance (NMR) tube for subsequent NMR spectral analysis.

NMR Data Acquisition and Processing

^{1}H NMR spectra were acquired at 25°C using the first transient of the Varian tnnoesy pulse sequence on a Varian 500 MHz Inova spectrometer equipped with a 5 mm HCN cold probe. Water suppression pulses were calibrated to achieve a bandwidth of 80 G. Spectra were collected with 128 transients and 8 steady-state scans using a 4 s acquisition time (48,000 complex points) and a 1 s recycle delay. Before spectral analysis, all free induction decays were zero-filled to 64,000 data points and line broadened to 0.5 Hz. The methyl singlet produced by DSS internal standard was used for chemical shift referencing (set to 0 ppm) and for quantification. Spectra were manually processed and 64 polar milk metabolites (including the 6 HMOs used for phenotype determination) were identified and quantified using Chenomx NMRSuite version 8.1 (Chenomx Inc., Edmonton, AB, Canada).

HMO Phenotype Determination

The HMO phenotype was determined based on the presence or absence of six specific milk oligosaccharides (2'FL, 3FL, LNFP I, LNFP II, LNFP III, and LDFT) in the NMR spectra that were identified and quantified from an NMR spectral library created through the analytical preparation of commercially available HMOs as previously described (21). In this study, the limit of detection was set to 20 μM for these compounds based on the ability to clearly observe spectral peaks of these HMOs over noise in the spectra generated from the Varian 500 MHz Inova spectrometer. Detection of both 2'FL and LNFP I in milk resulted in phenotype assignment as Se+, otherwise Se–. When LNFP II, 3FL, LDFT, and LNFP III were visible in the NMR spectra, the phenotype was assigned as Le+, otherwise Le–.

Statistical Analysis

Statistical computing and graphical generation were performed using the R (version 3.5.2) programing environment. Prior to principal component analysis (PCA), generalized log transformation [defined as $\log_2(1+y)$ where y is the metabolite concentration] was applied to all metabolomics data. PCA was computed using the *prcomp* function in the *stats* package of R without scaling the transformed data, and the first two components were plotted.

Metabolomics data without log transformation was used to perform log2_Fold calculation according the following equation. Briefly, the mean concentration of each metabolite was first calculated for the Se–Le–, Se–Le+, and Se+ phenotypes (the Se+Le+ and Se+Le– samples were combined since there was only one Se+Le– sample). The mean concentration of each metabolite in the Se–Le– (or Se–Le+) groups was divided by the mean concentration of the same metabolite in the Se+ group to calculate the ratio between Se–Le– (or Se–Le+) and Se+ phenotypes. To ensure metabolites were expressed in the same range, log2 transformation was applied. To decrease the chance of false discovery using FDR-corrected *p*-values (since most metabolites were significantly different using this method), we considered a log2 fold change cut off of ±1.5 as an indication of significance.

RESULTS

In total, 10 milk samples were collected from South African women 1 month after term delivery, of which 60% ($n = 6$) were Mixed Race, 20% ($n = 2$) were Black, and 20% ($n = 2$) were Caucasian. None of the women had atopic disease. An NMR spectrum annotated with HMO peaks is shown in **Figure 1A**. Multiple peaks of each HMO could be identified, with some overlapping with other metabolites in milk. The HMO phenotypes of the subjects was estimated by assessing the presence or absence of specific HMOs in the milk samples (**Table 1**), with examples of the NMR spectrum corresponding to each of the HMO phenotypes shown in **Figure 1B**. Samples where both 2'FL and LNFP I could be measured were assigned as Se+, while samples where these two HMOs could not be detected were designated Se–. No sample was detected with only one of the two HMOs. Se+ samples with the presence of LNFP II, 3FL, LDFT, and LNFP III were assigned as Se+Le+, otherwise they were assigned as Se+Le–. Se− samples with detectable levels of LNFP II, 3FL, and LNFP III were classified as Se–Le+, and for those without these three HMOs as Se–Le–. Out of 10 samples analyzed, three samples were designated Se–Le–, as none of the six targeted HMOs was detected in any of these samples. Additionally, the area under the peak for the three FUT 3-catalyzed HMOs (LNFP II, LNFP III and 3FL) were higher in milk from Se–Le+ mothers compared to milk from Se+Le+ mothers (**Figure 1B**).

To evaluate whether the milk metabolome was different among the HMO phenotypes, 58 quantified polar metabolites (excluding the HMOs resulting from FUT2 and FUT3)

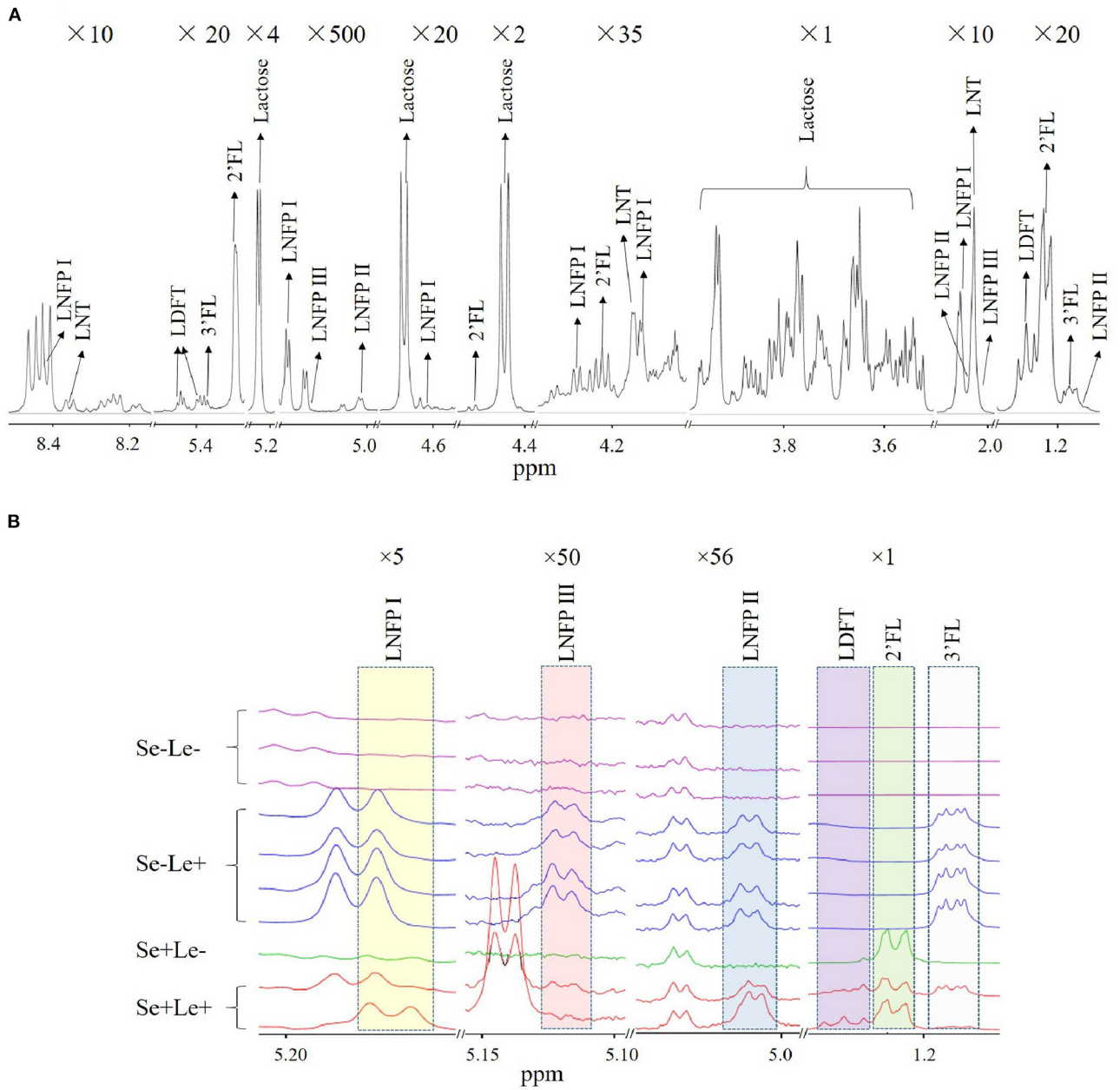

FIGURE 1 | Identification of HMOs in human milk NMR spectra. **(A)** Multiple peaks of each HMO are shown in 10 different chemical shift regions at various vertical scales to illustrate characteristic peaks associated with identified HMOs. Magnification is indicated at the top of each segment. **(B)** Comparison of NMR spectra of milk between individuals with putative differences in Se and Le status.

were examined and compared. **Figure 2A** shows a principal component analysis (PCA) of milk metabolites of women from the identified HMO phenotypes. Separation along PC1, which explained 48.7% of the variance, revealed a difference between the Se–Le– group and all other-12 groups. Along PC2, which explained 15.0% of the variance, separation based on Se status was observed. As there was only one sample identified as Se+Le–, and it did not separate from the Se+Le+ samples in the PCA plot (**Figure 2A**), it was combined with the Se+Le+ samples (Se+ samples) in further analyses.

In order to further compare milk metabolites among groups, the fold/ratio of metabolite concentrations in milk from Se–Le– and Se–Le+ mothers relative to milk from Se+ mothers were calculated (**Figure 3**). In terms of the

TABLE 1 | Proposed synthetic pathways of the principal fucosyloligosaccharides used to identify Secretor (Se) and Lewis (Le) phenotypes based on their presence/absence in the 10 milk samples.

Subject	Starting structure	LNT	Lactose	LNT	LNnT	Lactose	2'FL	Proposed HMO phenotype
	HMO phenotype	Se+		Le+				
	HMO	LNFP I	2'FL	LNFP II	LNFP III	3FL	LDFT	
1		bld	bld	bld	bld	bld	bld	Se–Le–
2		√	√	√	√	√	√	Se+Le+
3		bld	bld	√	√	√	bld	Se–Le+
4		bld	bld	√	√	√	bld	Se–Le+
5		bld	bld	bld	bld	bld	bld	Se–Le–
6		bld	bld	√	√	√	bld	Se–Le+
7		bld	bld	√	√	√	bld	Se–Le+
8		bld	bld	bld	bld	bld	bld	Se–Le–
9		√	√	bld	bld	bld	bld	Se+Le–
10		√	√	√	√	√	√	Se+Le+

LNFP I, lacto-N-fucopentose I; LNFP II, lacto-N-fucopentose II; LNFP III, lacto-N-fucopentose III; LNT, lacto-N-tetraose; LNnT, lacto-N-neotetraose; LDFT, lactodifucotetraose; 2'FL, 2'fucosyllactose; 3FL, 3-fucosyllactose; Se, secretor; Le, Lewis; √, Detected; bld, Below limit of detection. HMO concentrations below 20 μM were considered below the detection limit.

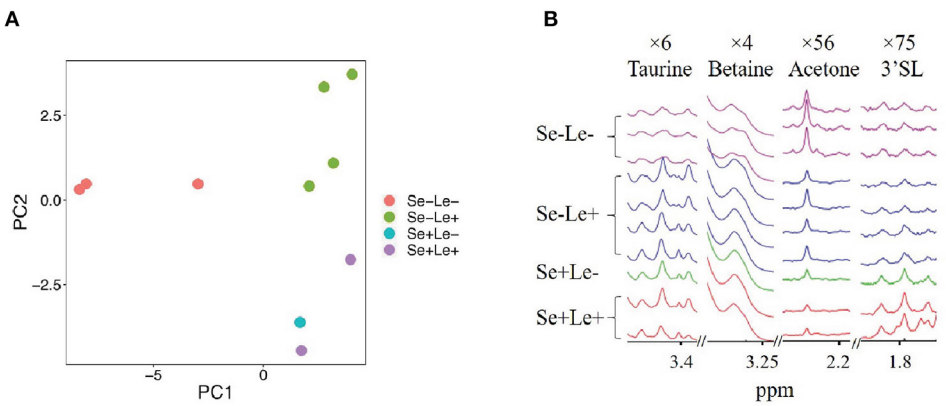

FIGURE 2 | Comparison of non-HMO milk metabolites between milk from women with different Se and Le status. **(A)** Principal component analysis of metabolites not used for HMO phenotype assignment. **(B)** Comparison of some non-HMO milk metabolites between Se-Le- and other phenotypes. Magnification is indicated at the top of each segment.

oligosaccharides and their metabolites, 3'galactosyllactose, 3'SL, fucose, and LNnT were between 2- and 10-fold lower in milk samples from Se–Le– and Se–Le+ compared to Se+ mothers. Galactose was 6 and 1 times higher in milk samples from Se–Le– and Se–Le+ mothers, respectively, compared to samples from Se+ mothers. For metabolites associated with energy metabolism, samples from Se–Le– milk were approximately 4 times higher in creatine phosphate, 12 times higher in creatine, 4 times higher in creatinine, 5 times higher in citrate, 6 times higher in pyruvate, and 10 times higher in succinate compared to Se+ milk, while these metabolites were similar in concentration between milk from Se–Le+ and Se+ mothers.

Milk from Se–Le– mothers also differed with respect to free amino acid concentrations compared to milk from Se+ and Se–Le+ mothers. Arginine, asparagine, glycine, leucine, isoleucine, lysine, and tyrosine were 2–4-fold higher in milk samples from Se–Le– compared to Se+. Interestingly, the fold difference of these amino acids in milk between Se–Le+ and Se+ samples was <2-fold. Carnitine was higher in milk from both the Se–Le– (∼4 fold) and Se–Le+ (∼2 fold) groups compared to Se+. Alanine, glutamate, glutamine, taurine, and betaine were all between 2- and 5-fold lower in the Se–Le– group compared to the Se+ group, while they were similar in concentration between the Se–Le+ and Se+ groups. Aspartate was also 2-fold lower in milk from both Se–Le– and Se–Le+ samples compared to Se+.

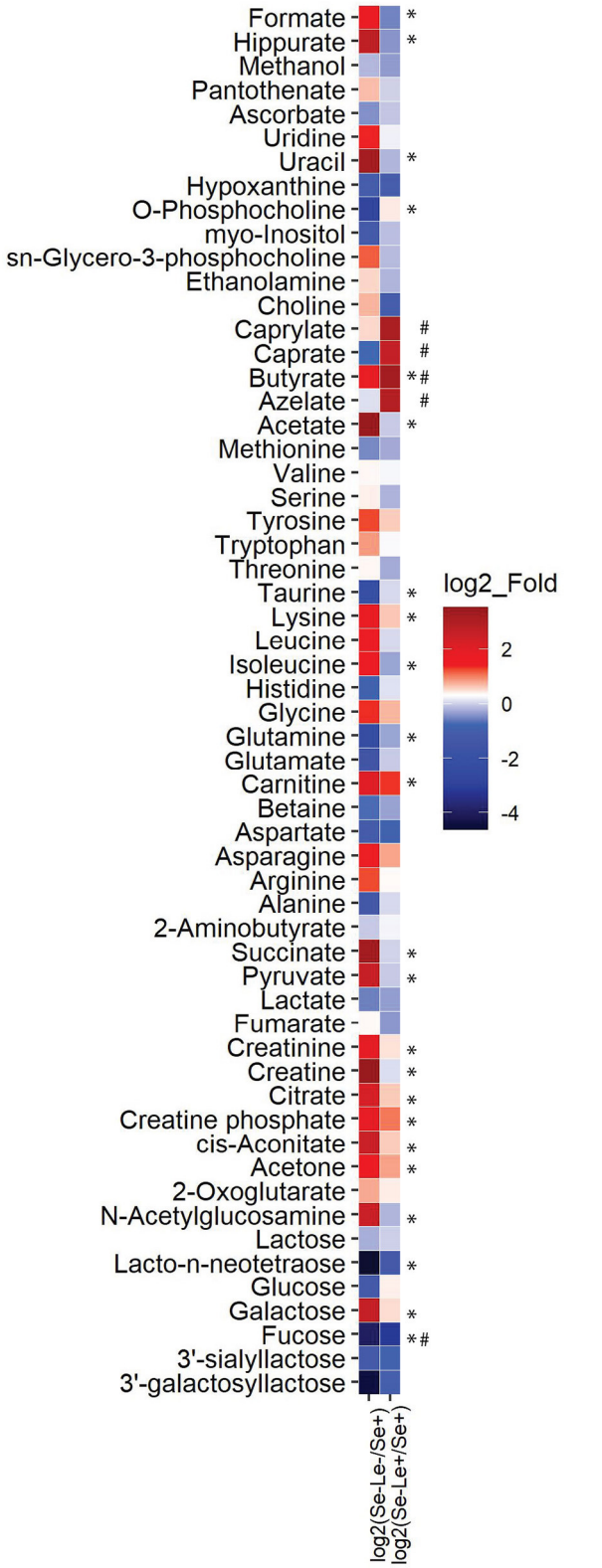

FIGURE 3 | Fold difference of metabolite concentrations in milk from Se–Le– and Se–Le+ mothers relative to Se+ mothers. The mean concentration of each

(Continued)

FIGURE 3 | metabolite was calculated for all groups and the means of the Se–Le– and Se–Le+ groups were divided by the mean of the metabolite concentration from the Se+ groups to determine the ratio relative to the Se+ groups. The ratio values were then log2 transformed. Log2_fold values over 1.5 or below −1.5 are indicated in the figure. * log2_fold over 1.5 or below −1.5 when comparing Se–Le– to Se+ samples. # log2_fold over 1.5 or below −1.5 when comparing Se–Le+ to Se+ samples.

Free fatty acids and associated metabolites such as acetate, choline, and sn-glycero-3-phosphocholine were 12-, 2-, and 2-fold higher, respectively, in the Se–Le– group compared to samples from Se+ mothers. Azelate, butyrate, caprate, and caprylate were also 8-, 10-, 6-, and 9-fold higher respectively in the Se–Le+ group compared to Se+ samples. Additionally, butyrate was 3- and 10-fold higher in Se–Le– and Se–Le+ groups compared to Se+. O-phosphocholine was lower in the Se–Le– group (10-fold) compared to Se+. Representative peaks of taurine, betaine, acetone and 3'SL are shown in **Figure 2B**. Metabolite concentrations for each subject are shown in **Supplementary Table 1**.

DISCUSSION

Significant research has been undertaken to understand the impact of maternal secretor status and Lewis blood type on the milk glycome (12) and subsequent influence on infant health including their gut microbiota (26), susceptibility to rotavirus (15), allergy to bovine milk (27), and weight during the first 6 months (28). But no studies have focused on the metabolome of milk from the Se–Le– population due to its low prevalence.

In the current study, we found that all of the 6 fucosylated HMOs used to determine HMO phenotypes were below the detection limit of the instrument in Se–Le– samples. 3FL and LNFP III were previously reported to be present in milk from Le− women, which could potentially be due to the activity of FUT4, 5, 6, 7, and 9 enzymes (1). In the current study, neither of these HMOs was detected in Le− women, which may be due to the difference in detection methods. Mass Spectrometry can measure down to the picomolar level, whereas for spectra obtained from the Varian 500 MHz spectrometer used in this study, the limit of detection of these metabolites was 20 μM. It remains to be determined if oligosaccharides present in milk below 20 μM would have a significant impact on infant health.

Other HMOs and related metabolites such as 3′galactosyllactose, 3′SL, fucose, galactose, and GlcNAc also trended different within the HMO phenotypes, indicating other factors influencing the glycome of human milk (**Figure 3**). 3′SL (**Figure 2B**), lower in both Se–Le– and Se–Le+ groups compared to Se+ in this study, was reported to be similar in concentration in milk from Se+ and Se− women (14, 21) or even ∼20–56% higher in milk from Se− compared to Se+ women (18, 29). 6′-sialyllactose, which is not reported in the current study, was demonstrated to be significantly higher in milk from non-secretor women (14). Further studies

are needed to investigate if this is due to a preference of α-2, 6-sialylation / α-2, 3-sialylation or simply a difference amongst populations.

Pyruvate, citrate, cis-aconitate, and succinate, which are metabolites involved in the tricarboxylic acid (TCA) cycle, were higher in Se–Le– milk. Increased TCA cycling could indicate greater energy provision, and previous studies have speculated that a higher level of TCA intermediates in bovine milk compared to human milk may be to enhance growth (30, 31).

HMO biosynthesis is suggested to be an extension of lactose biosynthesis which occurs in the Golgi of the mammary gland epithelial cells (32). Therefore, inactivity of both α-1,2- and α-1,3/1,4- fucosylltransferases in Se–Le– women might profoundly impact mammary gland metabolism, and thus impact milk composition. Alanine, taurine, glutamine and glutamate are the most abundant free amino acids in human milk (21, 33), and these were all lower in the milk from the Se–Le– group compared to milk from the Se+ group. Higher free glutamate in bovine milk infant formula has been reported to decrease its intake (5). It could be that a lower level of glutamate in milk from Se–Le– women could increase milk intake by the infant to compensate for the low and less diverse HMO content. Branched chain amino acids (leucine and isoleucine) and lysine were higher in Se–Le– compared to Se+ milk. A similar pattern of free amino acids in human milk was seen in a previous study comparing high and low growth rate groups of premature infants (34), where a higher content of insulinotrophic amino acids and tyrosine was associated with faster infant growth.

Choline in the Se–Le– group was almost double the level in Se+ samples, while phosphocholine was one-tenth the level. A previous study showed a negative correlation between choline and phosphocholine in human milk (35), and a similar correlation was observed in this study. The origin of choline in milk is not completely understood. One study reported that breastmilk choline is related to maternal choline intake and genetic polymorphisms (36), while another study showed no difference in milk choline content based on maternal diet (37). Indeed, the betaine level in Se–Le– milk was 2-fold lower than that in Se+ samples, suggesting a possible lower conversion of choline to betaine. It could be that the difference in milk choline (and other metabolites) in the Se–Le– group compared to Se+ group could result in differences in milk lipid synthesis (38). Indeed, choline is an essential precursor of phosphatidylcholine and sphingomyelin, which are essential components of biological membranes and precursors for intracellular messengers such as ceramide and diacylglycerol (39). This would imply that the milk fat globule would be different in Se–Le– mothers since maternal phenotype will impact conjugated glycolipids in addition to HMOs (40). Differences in the milk fat would need to be assessed in a separate study.

Here, in this brief research report, we showed differences in the non-HMO milk metabolome between phenotypically Se–Le– mothers and Se–Le+, and Se+ mothers. These differences included metabolites related to energy metabolism, amino acids, and fatty acids. The current study is limited by the small sample size and the rarity of Se–Le– HMO phenotype. Factors such as the completeness of milk expression, time since last feed, time of the day during sample collection, and information on mother's diet were not collected; however, the impact of these factors on milk composition is negligible compared to the impact of genetics. Nonetheless, this study shows that the Se and Le status of the mother has an important role to play in the composition of non-oligosaccharide milk metabolites. Further research involving larger sample sizes should be done to confirm the findings, investigate the impact on milk lipid and proteins, and investigate potential biological consequences of Se–Le– milk on infant gut microbial succession and metabolism. This will help further unravel the link between human milk and infant health.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article are available at: https://www.ebi.ac.uk/metabolights/MTBLS1899.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Cape Town's Human Research Ethical Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ET, DG, DM, SP, ME, CJ, GW, NS, DC, and AK designed the study. ET collected samples. PK prepared samples and collected NMR data. AW and CS analyzed and interpreted the data. AW drafted the manuscript. AK and CS provided funding. All authors edited and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2020.576966/full#supplementary-material

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Comparing Response of Sheep and Cow Milk on Acute Digestive Comfort and Lactose Malabsorption: A Randomized Controlled Trial in Female Dairy Avoiders

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Background: Sheep milk (SM) is a possible alternate dairy source for those who experience digestive symptoms with cow milk (CM). While both the milks contain lactose, one of the causes for self-reported intolerance to CM, the composition of SM and CM also differs across proteins and fats, which have been shown to impact digestive processes.

Objective: To compare the acute digestive comfort and lactose malabsorption of SM to CM in female dairy avoiders.

Method: In a double-blinded, randomized cross over trial, 30 dairy-avoiding females (aged 20–30 years) drank 650 mL of SM or CM (each reconstituted from spray dried powder) following an overnight fast, on two separate occasions at least 1 week apart. Blood samples were collected for glucose and insulin assessment, and single nucleotide polymorphisms of the lactase (*LCT*) gene (C/T_{13910} and G/A_{22018}). Breath H_2 and visual analog scale (VAS) digestive symptom scores were recorded at fasting and regular intervals over 4 h after ingestion.

Results: Eighty percentage of study participants were lactase non-persistent (LNP; CC_{13910} and GG_{22018} genotype). Digestive symptoms, including abdominal cramps, distension, rumbling, bloating, belching, diarrhea, flatulence, vomiting, and nausea, were similar in response to SM and CM ingestion (milk × time, $P > 0.05$). Breath H_2 was greater after CM than SM (72 ± 10 vs. 43 ± 6 ppm at 240 min, $P < 0.001$), which may be due to greater lactose content in CM (33 vs. 25 g). Accordingly, when corrected for the lactose content breath H_2 did not differ between the two milks. The response remained similar when analyzed in the LNP subset alone ($n = 20$).

Conclusions: Despite a higher energy and nutrient content, SM did not increase adverse digestive symptoms after ingestion, relative to CM, although there was a reduced breath H_2 response, which could be attributed to the lower lactose content in SM. The tolerability of SM should be explored in populations without lactose intolerance for whom underlying trigger for intolerance is unknown.

Keywords: ovine milk, bovine milk, lactose intolerance, digestive comfort, dairy avoidance, milk intolerance, postprandial

INTRODUCTION

Dairy is a major source of essential nutrients including Ca (1), high quality proteins, micronutrients (K and Mg), and vitamins (riboflavin, vitamins B_{12}, vitamin A, thiamin), in many cultures (1, 2). Complete dairy avoidance may increase the risk of nutrient insufficiency contributing to low bone mineral density, metabolic bone disease, or metabolic syndromes (3, 4). Milk intake has declined over the last few decades, especially in developed countries (5), where adverse gastrointestinal symptoms are a common reason for avoidance (6, 7). This is often attributed to the lactose in cow milk (CM) (8), resulting in lactose malabsorption. Yet, those reporting intolerance to CM are not always diagnosed as lactose intolerant (9, 10), and more recent evidence is emerging that other milk components including the protein fraction may induce similar symptoms (8, 11). However, the majority of people who avoid dairy, do so due to self-reported perception of symptoms rather than a confirmed diagnosis of intolerance (6, 8).

CM is the predominant type of dairy consumed, dominating global milk production (12); however, non-bovine dairy sources have important traditional and cultural origins (13, 14), and are increasing in availability worldwide (15). The increasing awareness of dairy intolerances, cow's milk protein allergy and vegan dietary preferences have all influenced consumers to seek alternative milk substitutes (16). However, segments of the population with substantial nutritional requirements commonly obtained from milk, including infants, children (16), and the elderly (17), may struggle to obtain equivalent nutrient density from plant-based sources (18). Sheep milk (SM) is one alternative to CM, containing higher concentration of micronutrients (Ca and P) (19), and macronutrients (proteins and fats) compared to CM (14, 20). The lactose content in SM and CM do not differ substantially (14) though lactose content in SM may vary with season and lactation period (14, 21). The anecdotal evidence, cited by others, that non-bovine ruminant milks [e.g., goat (22) and sheep (23)] may be better tolerated compared to CM currently lacks clinical evidence.

The compositional along with physiochemical variation between CM and SM may contribute to differences in milk digestion between CM and SM (12, 14, 24, 25). In addition to the higher protein content in SM, the constituent proteins differ between ruminant species. There is a higher β/α_s-casein ratio in SM (24), compared to CM. This influences the casein micelle formation with higher mineralization and diameter (14) and lower hydration and colloidal stability, resulting in faster coagulation (24), in SM relative to CM. The coagulation of milk has been shown to delay gastric emptying (26), and may contribute to differences in digestion depending on milk composition. Additionally, SM proteins (including caseins) have different sequences than CM proteins (27). This may result in different peptide formation during digestion (28). These differences may have important implications for digestive comfort, as variation in β-casein peptide formation has reported impacts on gastrointestinal transit (6), and may affect lactose digestion and any resulting abdominal discomfort (29–31).

There are limited studies on SM composition and physiochemical properties compared to extensive studies in CM (24). As yet no studies have reported how these differences impact self-reported digestive comfort and lactose malabsorption, particularly in dairy avoiders. Thus, this study aimed to compare the digestive comfort and lactose malabsorption responses to SM in dairy avoiders relative to CM. Due to the compositional and physicochemical differences between SM and CM, we hypothesized that SM would be tolerated better and digested more easily than CM in dairy avoiders including those with lactose intolerance.

METHODS

Study Design

We conducted a double-blinded, cross over randomized control trial at the Liggins Institute, The University of Auckland between July and November 2018. The primary outcome of the study is reported elsewhere (32). The study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the New Zealand Health and Disability Ethics Committees (Reference no. 18/NTB/92). The trial was prospectively registered with the Australian New Zealand Clinical Trials Registry (ACTRN12618001030268). Written informed consent was obtained from eligible participants prior to the study commencement.

A total of 32 healthy young women aged 20–40 years with BMI 18–28 kg/m^2 were recruited using digital and printed advertisements. Two subjects withdrew prior to the completion of the study and were excluded from further analyses (**Supplementary Figure 1**). All participants self-reported dairy avoidance. Subjects with known dairy allergy, current or history of gastrointestinal, cardiovascular, or metabolic disease, consuming medications expected to interfere with normal digestive and metabolic processes like proton pump inhibitors,

antibiotics, or prebiotics (3 months prior to the study) were not eligible.

Study Procedures

Eligible participants were randomized to consume 650 mL of either SM or CM on two occasions at least 1 week apart. Randomization sequences were computer generated using www.randomizer.org. Both participants and investigators were blinded to the treatment identity and allocation was implemented through sealed envelopes.

Prior to the clinical visits, demographic information was collected, including irritable bowel syndrome (IBS) classification, objectively assessed using Rome III criteria (33). One day prior to the visits, subjects were advised to abstain from vigorous physical exercise, avoid dairy and fiber rich food, and were provided with a standardized low fat, low dietary fiber dinner after which they were to remain fasted from 10.00 p.m.

Upon arrival, fasting breath samples were collected and gastrointestinal symptomology was recorded using a visual analog scale (VAS). A venous cannula was inserted to collect fasting blood samples. Subjects then consumed 650 mL of milk within 10 min and were asked to report their liking and perceived identity of each milk. Following milk ingestion, breath samples were collected every 15 min until 2 h and hourly until 4 h, whereas gastrointestinal symptoms and blood samples were collected every 30 min until 2 h and hourly thereafter until 4 h.

Digestive Symptoms and Likeability (Visual Analog Scale)

The severity of the subjective digestive symptoms was scored on a 100 mm VAS, with 0 mm corresponding to "no symptoms" and 100 mm corresponding to the "the most severe symptoms imaginable." The sum of scores for abdominal cramps, rumbling, diarrhea, flatulence, and vomiting >70 out of 500 was indicative of lactose intolerance (34). The other symptoms recorded included abdominal distension, bloating, belching, fecal urgency, digestive comfort, gastric reflux, and nausea.

The likeability of the milk was assessed on a hedonic VAS scale, including taste, aftertaste, smell, visual appeal, and palatability, with "0" mm corresponding to "good" and 100 mm corresponding to "bad."

Breath Hydrogen Analyses

AlveoSampler Breath Test Kits were used to collect the breath samples which were then analyzed using a BreathTracker H2+ (Quintron, Milwaukee, WI, USA). Data were recorded as CO_2 corrected H_2 concentrations (ppm) as a measure of lactose malabsorption.

Glucose and Insulin Analyses

Venous blood was collected in EDTA vacutainers (Becton Dickinson & Company, Mount Wellington, New Zealand), and plasma was removed after centrifugation at 2,000 × g for 15 min at 4°C and frozen at −20°C prior to analyses. Plasma glucose and insulin were measured using a Cobas c311 clinical chemistry analyzer (Roche Diagnostics, Manheim, Germany) and Cobas e411 immunoassay analyzer (Roche Diagnostics, Manheim, Germany), respectively.

TABLE 1 | Composition of sheep and cow milk (650 mL).

Component	Cow milk	Sheep milk
Total Energy (kJ)	1649.3	2140.4
Fat (g)	21.3	33.4
Protein (g)	19.4	29.9
Lactose (g)	33.3	24.9
Total solids (g)	79.0	91.7

Compositional analyses of milk was performed using a MilkoScan FT1 (FOSS, Denmark) analyzer. Test drinks were prepared using 81 g of whole cow milk powder or 98 g of whole sheep milk powder, reconstituted in 585 mL of water to make a final volume of 650 mL.

Milk Treatments

CM powder was sourced from NZMP (New Zealand Milk Products, Fonterra Co-Operative Group, Auckland, New Zealand). SM powder was sourced from Blue River Dairy (batch no. F2125/HC08) and Spring Sheep Milk Company (batch no. MAN: NOV17-JAN18). Prior to weighing and reconstitution, the SM powders were mixed in a 1:1 ratio. All powders were stored at −20°C until use. The reconstituted SM had higher concentrations of proteins, total solids, total energy, and fats but lower lactose than CM (**Table 1**). The compositional analyses of the milk were performed by a MilkoScan FT1 (FOSS, Denmark) analyzer using the default milk mosaic software. Additional details of milk composition and analyses are described elsewhere (32).

Spray dried milk powder was reconstituted in water on the day prior to the clinical visit. Pre-weighed portions of CM powder (81 g) or SM powder (98 g) were reconstituted in 585 mL of filtered water heated at 30°C to make a final volume of 650 mL, shaken well and stored at 4°C overnight. The milks were served chilled in a transparent plastic bottle. The reconstitution was performed to match the typical solid content of each milk, with SM having higher solid content than CM.

The volume of 650 mL was chosen to exceed the volume of milk (250 mL) reported to be well-tolerated by those with lactose intolerance (35). This volume is similar to what has been used previously (30, 36–38) to evoke symptoms of digestive discomfort, while providing an appropriate quantity of protein and fat for assessment of the primary and secondary outcomes of the trial (32).

Lactase Persistence Genotyping

Lactase persistence and lactase non-persistence (LNP) in the study participants was determined using the iPlEX assay and MassARRAY® System (Agena Bioscience, SanDiego, USA) by Grafton Clinical Genomics (GCG, Auckland, New Zealand). Peripheral blood mononuclear cells (PBMCs) were isolated immediately from fasted whole blood collected in EDTA-containing blood collection tubes using a histopaque solution (Sigma- Aldrich, St. Louis, MO, USA) as previously described (39). The samples were stored at −80°C until DNA was extracted.

All prep DNA/RNA mini kit (Qiagen, Hilden, Germany) was used to isolate genomic DNA from PBMCs as per the manufacturer's protocol.

Subset Analyses

Breath H_2 and sum of symptoms (abdominal cramps, rumbling, diarrhea, flatulence, and vomiting) for lactose intolerance were also analyzed separately in the LNP subset as these individuals are more susceptible to lactose malabsorption and associated symptoms.

Statistical Analyses

A sample size of 30 was calculated for the primary outcome as described elsewhere (32). However, to provide an 80% power with alpha set at 5%, based on previously provided mean for nausea (8 mm vs. 15 mm) and a standard deviation of 9 mm, 26 subjects would be required (30). The impact of variation in milk composition on digestive symptoms has been previously reported acutely in the context of β-casein variants (30).

Individuals with fasting breath H_2 above 25 ppm were excluded for breath H_2 analyses and treated as outliers, as this is identified as the threshold for malabsorption of carbohydrates, and impacts the results of standardized breath hydrogen tests (40). Although best practice for hydrogen breath tests would require rescheduling the test (41), this was not provisioned for in the protocol as lactose malabsorption was a secondary outcome. The primary outcome was to compare the rate of digestibility of the proteins (32) which was not dependent on fasting breath H_2 below 25 ppm. Values missing completely at random were estimated using multiple imputations as the mean of 5 iterations. The incremental area under the curve (iAUC) was calculated using the trapezoidal method, correcting for baseline concentration. As lactose dose has been shown to affect breath H_2 concentrations (36, 42, 43), and given the difference in lactose content between the two milks, an adjusted breath H_2 concentration and iAUC were further calculated and analyzed accordingly to match the lactose content.

iAUC and hedonic likeability were analyzed between the two milks using Student's paired *t*-test. Frequency for identification of milk (CM or SM) was analyzed using Pearson's chi-square test (χ^2). Other outcomes with multiple factors were compared using repeated measures general linear model with milk and time compared within-subject; multiple comparisons were corrected using Sidak adjustment. Alpha was set at $P < 0.05$ for all tests. All statistical analyses were performed using SPSS version 25 (SPSS, IBM Corporation, Armonk, NY, USA).

RESULTS

Demographics

Thirty female participants aged 24.3 ± 1.3 years completed the study with anthropometric and biochemical values within a normal range. Participants self-identified as Caucasian (30%), Asian (37%), and South Asian (33%), with 40% being classified as IBS based on Rome III criteria. Based on lactase genotyping, 80% of the participants were LNP (CC_{13910} and GG_{22018}) and only 20% were LP (CT_{13910} or TT_{13910} and GA_{22018} or AA_{22018}) (**Table 2**). Four LNP subjects and one LP subject had a fasting breath $H_2 > 25$ ppm and were excluded from malabsorption analyses.

TABLE 2 | Baseline characteristics ($n = 30$).

Measures	Values[a]
Age, y	24.3 ± 1.3
BMI, kg/m^2	22.8 ± 0.9
Glucose (mmol/L)	4.8 ± 0.1
Insulin (μU/mL)	7.6 ± 0.6
Rome III IBS[b] *n* (%)	12 (40)
LNP (CC_{13910}/GG_{22018})[c] *n* (%)	24 (80)
Ethnicity	
Caucasian, *n* (%)	9 (30)
Asian, *n* (%)	11 (37)
South Asian, *n* (%)	10 (33)

[a] *Values presented as mean ± SEM or count (percentage) as indicated.*
[b] *IBS, Irritable bowel syndrome.*
[c] *LNP, Lactase non-persistence based on SNPs (single nucleotide polymorphisms) analyses.*

Milk Likeability and Identification

The frequency of identification reporting indicated that subjects were more likely to perceive CM as CM ($n = 19$) and SM as SM ($n = 21$) ($P = 0.010$, χ^2). There was no difference in the reported liking between SM and CM for taste, smell, palatability, aftertaste, and visual appeal ($P > 0.05$ each, respectively; **Supplementary Table 1**).

Digestive Symptoms in Response to SM and CM

There were no differences in the severity of lactose malabsorption associated subjective abdominal symptoms (sum of abdominal rumbling, cramping, flatulence, diarrhea, and vomiting) reported between the two milk types ($n = 30$; milk × time interaction, $P = 0.916$). Likewise, the iAUC did not differ between milks ($P = 0.559$). Regardless of milk type, individuals experienced an increase in lactose malabsorption associated symptoms following milk ingestion (main time effect, $P < 0.001$; **Figure 1**). Likewise, independent subjective digestive symptoms, including abdominal cramps, rumbling, bloating, belching, flatulence, fecal urgency, diarrhea, nausea, and vomiting, did not differ between milks (milk and time × milk interaction, $P > 0.05$ each, respectively; **Supplementary Figure 2**) but these symptoms increased following ingestion of either milk (main time effect, $P < 0.001$). No adverse events of vomiting were reported. Gastric reflux was not different from baseline for either milk (main time effect, $P = 0.305$).

Lactose Malabsorption

Regardless of milk type, breath H_2 increased postprandially ($n = 25$; main time effect, $P < 0.001$) but the increment was greater after CM compared to SM (milk × time interaction, $P = 0.013$; $P < 0.05$ *post-hoc* comparison between CM and SM at 30, 45, 60,

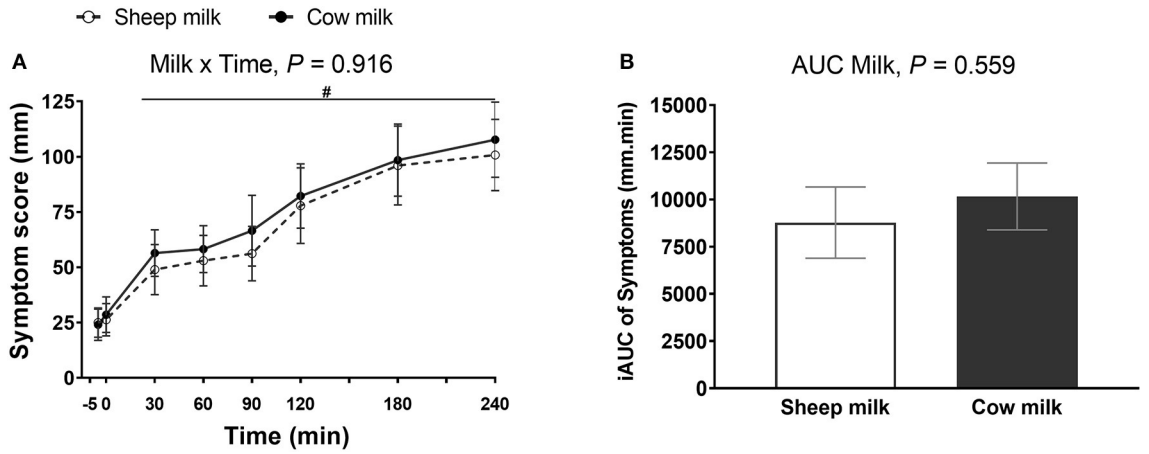

FIGURE 1 | Subjective VAS scores (sum of abdominal cramps, abdominal rumbling, flatulence, diarrhea, and vomiting) **(A)** at multiple timepoints and **(B)** 4-h incremental area under the curve (iAUC) following cow milk and sheep milk ingestion ($n = 30$). Values are presented as means ± SEM. Data for multiple time points were compared using repeated general liner model with milk and time compared within-subject and iAUC was compared using Student's paired *t*-test. There was no milk × time interaction, $P = 0.916$ and iAUC, $P = 0.559$. There was a significant time effect **(A)**, $P < 0.001$. # denotes indicated time points were significantly different from baseline.

75, 180, and 240 min) (**Figure 2A**). The AUC was also higher after CM compared to SM ($n = 25$; $P = 0.015$) (**Figure 2B**).

Given the difference in lactose content between the reconstituted milks (33 g in CM vs. 25 g in SM), breath H_2 was adjusted to match the lactose content. After lactose adjustment (considering the lactose content in SM was 33 g, the H_2 value for SM was multiplied by 33/25 = 1.32), breath H_2 did not differ between milks at any time point (milk × time interaction, $P = 0.069$) (**Figure 2C**), and the iAUC was no longer different between milks ($P = 0.131$) (**Figure 2D**). However, CM ingestion in general resulted in higher breath H_2 when corrected for lactose content compared to SM (main milk effect, $P < 0.001$).

LNP Subset Analysis of Digestive Symptoms and Lactose Malabsorption

Given lactose malabsorption contributes to symptoms of intolerance and is highly linked to LNP status, subset analysis of symptoms and malabsorption were completed on LNP subjects. LNP subjects ($n = 20$) showed the same patterns of response as the total sample set. Digestive symptoms did not differ between milks ($n = 24$; milk × time interaction, $P = 0.750$; iAUC, $P = 0.365$) although increased with time regardless of milk type (main time effect, $P < 0.001$) (**Supplementary Figure 3**). Breath H_2 was higher after CM compared to SM ($n = 20$; milk × time interaction, $P = 0.009$ and iAUC, $P = 0.030$) but after lactose adjustment, it did not differ (milk × time interaction, $P = 0.520$ and iAUC, $P = 0.135$; **Supplementary Figure 4**).

Plasma Glucose and Insulin Analyses

The plasma glucose and insulin responses did not differ between the milk types. The iAUCs for glucose after ingestion of CM compared to SM were −204 ± 21. vs. −168 ± 23 mmol·min/L, and the iAUCs for insulin were 2,400 ± 186 vs. 2,377 ± 171 μU·min/mL ($P > 0.05$, each, respectively).

DISCUSSION

SM is a nutritionally rich alternative to CM which may be digested differently, owing to its unique composition and physiochemical properties. Thus, this study investigated digestive comfort and lactose malabsorption following ingestion of SM compared to CM. In contrast to the hypothesis, subjective digestive symptoms did not differ between the two milks, but breath H_2 was raised to a greater extent by CM than SM. While the dairy avoiders included in this study were largely LNP, indicating likely lactose intolerance, subset analysis of LNP subjects only indicated that the symptoms and lactose malabsorption responses were the same, as for the entire study group.

In these dairy avoiders, ingestion of either milk resulted in increased subjective digestive symptoms. Digestive symptoms reported after milk ingestion are influenced by several factors including compositional variations of milk [lactose (44), fats (45) and protein content (30)], intestinal transit or gastric motility (46), colonic flora (47), and visceral sensitivity (48). Higher fat (45) and energy content (49) are known to slow gastric emptying and fat specifically has been shown to increase the jejunal transit time (50). Variation in milk protein sequences [such as those between SM and CM (28)] and protein integrity [hydrolyzed or whole protein (37)], impact digestive products (28) and gastric emptying (37) or incretin responses (51). Other protein-related effects, such as those observed with A1 β-casein, have also been shown to impact lactose malabsorption and related symptoms (30), however the mechanisms for this are not clear. It is known that delayed intestinal transit of lactose is responsible for reduced malabsorption and symptoms of intolerance, as observed during

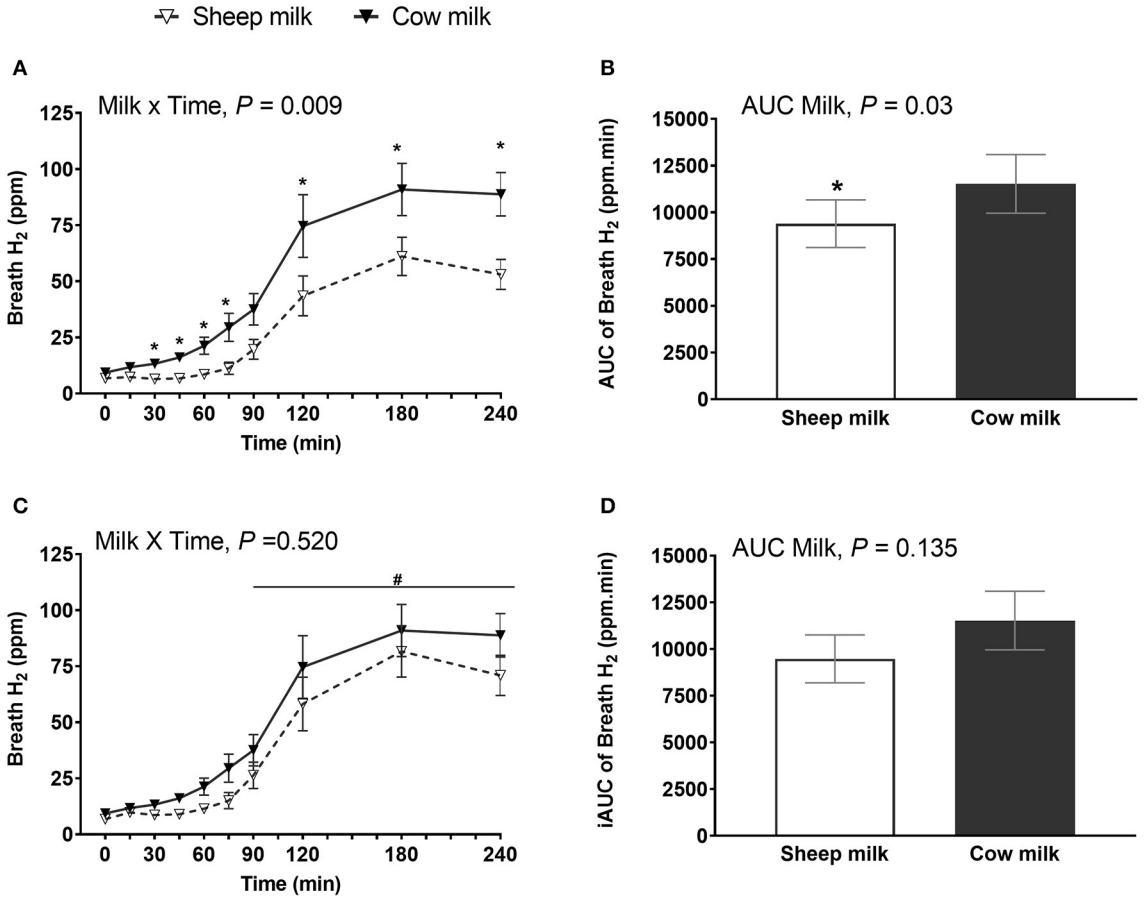

FIGURE 2 | Breath hydrogen following sheep milk and cow milk ($n = 25$, after removal of outliers) at multiple time points **(A,C)** and 4-h incremental iAUC **(B,D)**, before **(A,B)** and after lactose adjustment **(C,D)** i.e., considering equal lactose content in both SM and CM. Values are presented as means ± SEM. Data for multiple time points **(A,C)** was compared by repeated measures general linear model with milk and time compared within-subject and iAUC **(B,D)** was compared using Students paired *t*-test. Prior to lactose adjustment **(A,B)** there was significant milk x time interaction, $P = 0.013$ and iAUC, $P = 0.015$. After lactose adjustment **(C,D)**, there was no milk x time interaction, $P = 0.069$ and iAUC, $P = 0.131$. * denotes $P < 0.05$, indicated timepoints were different between the milks after *post-hoc* correction. and iAUC was different between the milks. There was a significant time effect **(C)**, $P < 0.001$. # denotes indicated time points were significantly different from baseline.

pregnancy (52). Individual features like visceral hypersensitivity may also induce digestive symptoms in lactose malabsorbers (48), or the onset of the symptoms and severity may depend on the colonic bacteria and their fermentation pathways (47). Although this study did not directly assess gastrointestinal function, the similar subjective digestive symptoms between the milks suggest that in a group that is largely lactose malabsorbers, there may not be large differences.

After equal volumes, breath H_2 was increased to a greater extent following CM than SM ingestion, indicating higher lactose malabsorption after CM. The present study used reconstituted powdered milk to match the solid content of fresh liquid milk which resulted in a 24% lower lactose content in SM than CM. While some studies report similar lactose content in SM and CM (14, 53), others have reported lower lactose content in SM (54), similar to the content in current study. The composition of SM varies seasonally with lower lactose content at the end of lactation (14, 21). Previous studies show that breath H_2 may depend on the dose of lactose ingested (55, 56), so the H_2 concentration was further adjusted to match lactose doses between milks. After the adjustment, overall breath H_2 (iAUC) was not different between the two milks. It is important to note that although breath H_2 may depend on the dose of lactose (55, 56), the increment may not be directly correlated (57), especially when milk is used as a substrate (58). The rise in breath H_2 is influenced by factors including the complexity of the food matrix (36, 58), inter-individual variations, gut microflora (47, 59, 60), and gastrointestinal transit (46). The complex interaction of all these factors precludes calculation of compensatory adjustment of breath H_2. The adjustment of breath H_2 concentrations for specific substrates or doses is not a standardized practice (42), and given that lactose dosing may even require adjustment for body weight (61), may not be a straightforward calculation. Although the current study showed lower lactose malabsorption with SM, differences in lactose digestion following SM should be investigated within the upper

range of lactose content naturally occurring in SM, which may be more closely matched to CM.

Dairy intolerance is mainly attributed to lactose, with characteristic symptoms of diarrhea, flatulence, abdominal rumbling, cramping, and vomiting (34, 44). Despite lower lactose content of SM resulting in lower lactose malabsorption, no impact on subjective digestive comfort was observed. This supports evidence in the literature showing that although lactose malabsorption may result in abdominal discomfort (62), the severity of digestive symptoms is not always correlated to lactose malabsorption (57). As such, lowering the lactose content in milk may improve lactose malabsorption but not tolerance. However, the majority of subjects in the current study were LNP as defined by *LCT* gene SNPs C/T_{13910} and G/A_{22018}, and were lactose malabsorbers as reflected in their breath H_2 concentrations. Most LNP individuals tolerate low doses of lactose (<12 g per serving) (44, 62) but above this dose report symptoms. As this study used a high volume of milk (650 mL), the lactose content was sufficient (>12 g) to induce symptoms in lactose intolerant individuals despite the compositional variations. However, it should be noted that such a high volume is more than twice a usual serving size of milk—this high volume of milk may have resulted in more severe symptoms than might be expected for those with sensitivity to milk. For SM with a higher nutrient density, this effect may have been greater. It is unclear whether a more usual serving size may have revealed differences in the severity of subjective symptoms between SM and CM. Indeed, the underlying intolerance explains the prevalence of symptoms associated with lactose intolerance reported following ingestion of both milks. Furthermore, these symptom and breath H_2 findings remained the same when analyzed with LNP subset, highlighting that the current study is mainly reflective of lactose mediated intolerance, but may not be representative of SM tolerance with a non-lactose mediated dairy intolerance. Thus, further studies are required to determine whether individuals without lactose malabsorption experience different digestive tolerance to SM relative to CM, and whether typical serving sizes of SM are tolerated differently to CM.

Dairy intolerance has been reported to be caused by characteristics of milk other than lactose. Intolerance can occur in the absence of lactose malabsorption (30, 63) and has been attributed to sensitivity to bioactive peptides released during milk digestion (64). In this case, it may have been expected that the lack of some milk proteins, like A1 β-casein in sheep milk (27), may have contributed to less discomfort than CM, as A1 β-casein has been implicated in digestive discomfort (64). Yet, the proportion of A1 β-casein in the CM may have been less than expected, as New Zealand conventional bovine herds have been reported to produce milk with only ∼22% A1 β-casein (30), and may in part explain a lack of difference in subjective symptoms. Indeed, the variety of bioactive peptides in milk, many with known links to IgE and non-IgE-mediated immune responses (65), could have contributed to symptoms in the current study. Although those with known milk allergy were excluded, no comprehensive testing of milk protein sensitivity was conducted. Differing or modification of dairy structures are known to influence digestion and resulting physiological responses (66); homogenization and pasteurization (impacting protein and fat structures), has been reported to aggravate intolerance symptoms, particularly in those with lactose malabsorption (38). Therefore, species-specific physiochemical differences independent of lactose, including density, may have influenced tolerance to SM and CM in the current study. Further, the large variability in symptom responses across subjects highlights the diverse experience of "dairy avoiders" in response to milk. This aligns with the recent description of differing symptoms traits and severity in differing types of dairy intolerance (30), and suggests that detection of differences in comparative symptoms between SM and CM may be more clear in specific subsets of dairy intolerance (e.g., not lactose intolerant) with a common pathophysiology Besides, digestive comfort and lactose malabsorption, SM provides more branched chain amino acids which may benefit individuals with increased protein requirements (32).

The current study included only young female participants, who were largely lactose intolerant, and may not represent all people who avoid bovine dairy or seek alternatives. The prevalence rate of gastrointestinal symptoms (67) and IBS (68) are higher in females than males which may suggest gastrointestinal symptoms reported in the present study could be overestimated. In addition, age related physiological changes in the gastrointestinal tract (69) may impact the digestive process and digestive symptoms in the elderly. Thus, generalizability of these findings to males, older populations, or tolerant individuals is limited, warranting further studies considering both sexes of different age groups and including a control dairy-free comparator. The pain tolerance in females may also vary with the stage of the menstrual cycle (70) and with a wash out of only 1 week, which given the possibility of a relationship between menstrual phase and digestive symptoms, this may have impacted on the symptoms reported (71). However, this was not considered in the present study. Furthermore, despite a trend of a higher digestive symptoms after CM compared to SM, these differences were not significant. In part this may be due to the relatively small sample size and the wide variations of self-reported subjective symptoms experienced. These findings highlight the need to better understand the spectrum of intolerance pathophysiology in non-lactose dairy intolerant individuals.

Perceptual differences between milks may have also mediated subjective symptom reports in this study. Milks were blinded but not masked, and participants were able to differentiate SM and CM by taste. However, as this had no impact on likeability scores, there may not have been a notable influence on symptoms. Further, although VAS are validated for pain (72), these scales may not have captured symptoms of discrete events, like vomiting and diarrhea, adequately. In the current study, no adverse events of vomiting were recorded, yet reports by VAS showed high variability between subjects—possibly reflecting associated feelings, rather than a discrete event. As such, these symptoms may be poorly suited for timed VAS reporting, reflected in the high variability between subjects. This is supported by literature showing that feelings

of nausea, rather than vomiting itself, are validated by VAS (73). This study only explored acute digestive responses. Longer term studies may show different kinetics, as habitual dairy ingestion may improve tolerance to lactose (74). Furthermore, the compositional discrepancies across macro (44, 45) and micronutrient contents (75) in the reconstituted milk relative to their naturally occurring counterparts may have influenced digestion. Despite the variation in dairy both seasonally and across species/products (14), the current study serves to provide initial insights into acute digestive response following an equal volume of SM compared to CM.

In summary, dairy avoiders, who were largely intolerant to lactose in CM, experienced similar digestive symptoms following an equal volume of SM and CM, despite higher lactose malabsorption after CM ingestion. This highlights that the digestive discomfort of milk intolerance is complex and impacted by more than just lactose. Further, tolerability of SM over CM should be additionally explored in populations without lactose intolerance but who still experience adverse symptoms associated with milk ingestion, for whom the underlying trigger of intolerance may be unclear.

DATA AVAILABILITY STATEMENT

The datasets generated for this article are not readily available because approval has not been granted by subjects. Requests to access the datasets should be directed to a.milan@auckland.ac.nz.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by New Zealand Health and Disability Ethics Committees. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AS conducted research, analyzed data, performed statistical analyses, and wrote the first draft of the paper. LS, LD, and DC-S designed research and reviewed the paper. PS conducted research. AM designed and conducted research, wrote the paper, and had primary responsibility for final content. All authors approved the final version of the manuscript for submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.603816/full#supplementary-material

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Addition of Dairy Lipids and Probiotic *Lactobacillus fermentum* in Infant Formulas Modulates Proteolysis and Lipolysis With Moderate Consequences on Gut Physiology and Metabolism in Yucatan Piglets

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Breast milk is the gold standard in neonatal nutrition, but most infants are fed infant formulas in which lipids are usually of plant origin. The addition of dairy lipids and/or milk fat globule membrane extracts in formulas improves their composition with beneficial consequences on protein and lipid digestion. The probiotic *Lactobacillus fermentum* (Lf) was reported to reduce transit time in rat pups, which may also improve digestion. This study aimed to investigate the effects of the addition of dairy lipids in formulas, with or without Lf, on protein and lipid digestion and on gut physiology and metabolism. Piglets were suckled from postnatal days 2 to 28, with formulas containing either plant lipids (PL), a half-half mixture of plant and dairy lipids (DL), or this mixture supplemented with Lf (DL+Lf). At day 28, piglets were euthanized 90 min after their last feeding. Microstructure of digesta did not differ among formulas. Gastric proteolysis was increased ($P < 0.01$) in DL and DL+Lf (21.9 ± 2.1 and 22.6 ± 1.3%, respectively) compared with PL (17.3 ± 0.6%) and the residual proportion of gastric intact caseins decreased ($p < 0.01$) in DL+Lf (5.4 ± 2.5%) compared with PL and DL (10.6 ± 3.1% and 21.8 ± 6.8%, respectively). Peptide diversity in ileum and colon digesta was lower in PL compared to DL and DL+Lf. DL and DL+Lf displayed an increased ($p < 0.01$) proportion of diacylglycerol/cholesterol in jejunum and ileum digesta compared to PL and tended ($p = 0.07$) to have lower triglyceride/total lipid ratio in ileum DL+Lf (0.019 ± 0.003) as compared to PL (0.045 ± 0.011). The percentage of endocrine tissue and the number of islets in the pancreas were decreased ($p < 0.05$) in DL+Lf compared with DL. DL+Lf displayed a beneficial effect on host defenses [increased goblet cell density in jejunum ($p < 0.05$)] and a trophic effect

[increased duodenal ($p = 0.09$) and jejunal ($p < 0.05$) weights]. Altogether, our results demonstrate that the addition of dairy lipids and probiotic Lf in infant formula modulated protein and lipid digestion, with consequences on lipid profile and with beneficial, although moderate, physiological effects.

Keywords: infant formula, milk fat, probiotic, digestion, intestinal physiology, glucagon-like peptide-1

INTRODUCTION

Early nutrition is essential to ensure optimal infant growth and development, especially regarding the digestive functions, which are immature at birth (1). While human milk is recognized as the gold standard for infant nutrition, a large proportion of them are formula-fed (2). Infant formulas have been much improved over the last decades regarding their nutritional content; however, differences remain between infant formulas and human milk regarding their non-nutritive composition, i.e., protein/lipid structure, oligosaccharide, and bacterial content (3).

At the supramolecular level, human and bovine milk fat is organized under its native form into dispersed globules enveloped in a biological membrane called *milk fat globule membrane* (MFGM). However, the step of homogenization during infant formula processing is conducted to uniformly distribute submicronic droplets and impact the compositional and organizational architecture of the lipid–water interface (3).

At the molecular level, the triacylglycerol structure, i.e., the regiodistribution of the fatty acids, impacts fatty acid absorption. This is of particular importance for palmitic acid, a major fatty acid in human milk. In the latter, more than 70% of the palmitic acid is located in the sn-2 position, while this is true for <20% of the palmitic acid from palm oil, a major fat source for infant formula based on plant oil. Dairy lipids present an intermediate profile, with 40–45% of palmitic acid in this inner position (4, 5). Human pancreatic lipase is sn-1,3 specific, and when palmitic acid is located at these positions, its absorption as non-esterified fatty acids is reduced as it tends to form calcium soap that is excreted in stool. On the contrary, when palmitic acid is esterified in sn-2 position, pancreatic lipolysis results in the formation of water-soluble palmitoyl-monoglycerol, which is well-absorbed. Accordingly, palmitic acid and calcium have been shown to be better absorbed in breastfed infants as compared to infants fed plant oil–based formulas (6).

A better understanding of the impact of the formula matrix effect onto its digestion in the infant gastrointestinal tract is therefore essential to optimize their formulation and improve the health of formula-fed infants. However, ethical and financial constraints of clinical studies limit knowledge. *In vitro* systems designed to mimic infant digestion, although relevant, cannot reproduce all the biological complexity of the digestive tract, such as digestion and absorption, hormonal feedbacks, neural interactions, host and microbe interactions, and microbe and microbe interactions. Piglet was identified as a more appropriate *in vivo* model to study infant digestion (7). In this model, the addition of DLs and MFGM extracts was shown to have structural and biochemical consequences on infant formula digestion, decreasing small intestinal digestion of casein and β-lactoglobulin and leading to more numerous β-casein peptides in intestinal contents, but increasing small intestinal lipid digestion (8). Besides, the administration of MFGM extracts with probiotic *Lactobacillus fermentum CECT 5716* (Lf), lactic acid bacteria originally isolated from human milk (9), was shown to decrease the whole gut transit time of rat pups (10). Interestingly, this effect was not observed when MFGM extracts were provided alone, suggesting a potential role of probiotic Lf on infant formula digestion. Whether all these features remain true when DLs without additional supply of MFGM are provided alone or with the Lf probiotic in infant formulas is unknown.

It was previously demonstrated that the addition of dairy lipids to replace partially plant lipids and without additional supply of MFGM extracts and probiotic Lf in infant formula had a beneficial impact on the intestinal endocrine function later in adulthood, enhancing cecal GLP-1 content and GLP-1 meal-stimulated secretion in adult mini-pigs (11). However, the impact of infant formula containing dairy lipids with or without probiotic Lf on the piglet intestinal endocrine function and the consequences on pancreas maturation and glucose homeostasis are currently unknown. Addition of dairy lipids in the infant formula, inducing modification in proteolysis and the presence of peptides more distally in the intestine as described above, could stimulate GLP-1 secretion by the distal L-cells in ileum (12, 13), an effect that could be enhanced if transit time is also decreased as described above with Lf.

Our hypothesis was that the addition of dairy lipids and probiotic Lf in infant formulas could impact their digestion and consequently piglet lipid and protein metabolism. The objective of this study was to compare the digestion of infant formulas containing dairy lipids in the presence or absence of probiotic Lf to a reference formula containing only plant lipids and to evaluate their metabolic impact in infant formula-fed piglets.

METHODS

Ethical Approval

The present study was designed and conducted in compliance with the current ethical standards of the European and French guidelines (directive 2010/63/EU and decree 2013-118, respectively). The ethics committees of CREEA (Rennes Committee of Ethics in Animal Experimentation) and of France's Ministry of Higher Education and Research approved the protocol (authorization #2016011111546978). Animals were observed daily throughout the experimental protocol to ensure their welfare, and they received no medication or antibiotic treatment.

Animals and Study Design

The study design has already been published in Lemaire et al. (11). A total of 27 female and male (11 and 16, respectively) Yucatan piglets (Saint-Gilles, France) were used in three replicates. One animal was excluded from the analyses because of health issues. Piglets were separated from their sow at postnatal day (PND) 2 and housed in individual stainless-steel metabolic cages. They were fed one of the three experimental formulas with an automatic milk feeder as previously described (14) until weaning, i.e., at PND28. To account for litter-to-litter variation, three piglets with a body weight (BW) close to the mean birth weight of the litter were selected from each litter and assigned to one of the three formulas. Allocation to formulas was balanced between groups for birth weight, BW at PND2, and sex. Formulas were rehydrated each day at 20% dry matter extract in water before distribution. The formula was allocated in 10 meals automatically distributed during the day. BW was measured twice a week, and feeding amounts were adjusted accordingly. The daily net energy offered was 1450 kJ/kg $BW^{0.75}$. Formula intake was automatically recorded for each meal. The average daily volume of formula intake was 220 ± 4 mL/kg $(BW)^{0.75}$. Piglets were euthanized at PND28, and tissues were collected and weighed.

Diets

Formulas were manufactured by Lactalis (Retiers, France) and adapted to meet piglet energy and protein requirements. The three formulas had the same energy, protein, lipid, and carbohydrate levels. They differed by the lipid origin, only plant lipids ($n = 9$) vs. half-half plant lipids and dairy lipids ($n = 9$), and the supplementation with Lf (DL+Lf, $n = 8$), as described in **Table 1**. Dairy lipids came from the cream, which may contain some residual MFGM that may account for 2–6% of the fat mass (15), but there was no addition of specific MFGM. The experimental formulas contained higher amounts of protein and lipid and a lower amount of lactose than a standard human infant formula in order to meet the piglet requirement. Lipid:protein and linoleic acid:α-linolenic acid (ω6:ω3 = 6–7) ratios were kept similar to those found in commercial infant formulas. The formulas were based on a mixture of skim milk and whey protein concentrate powders to reach a casein:whey proteins ratio of 30:70 wt/wt.

TABLE 1 | Composition of infant formulas.

g/100 g of powder	PL[a]	DL[a]	DL+Lf[a]
Proteins	17.8	17.9	17.9
Lipids	43.6	44.7	44.6
Carbohydrates	33.1	32.3	32.2
Minerals	3.5	3.4	3.4
Energy (kJ)	2476	2506	2501
Lactobacillus fermentum CECT 5716 (Lf)	—	—	1.9E+08
Phospholipids[b]	0.40	2.52	2.52
Cholesterol[c]	0.004	0.054	0.054

[a] *Formulas contained as lipids either only plant lipids (PL), a half-half mixture of plant and dairy lipids (DL), or a half-half mixture of plant and dairy lipids supplemented with Lf (DL+Lf). Formulas were rehydrated at 20% of dry extract. Lipid sources of the PL formula were palm oil (71.7%), rapeseed oil (23.2%), and refined sunflower oil (5.1%); those of the DL and DL+Lf formulas were cream (53.4%), rapeseed oil (21.1%), refined sunflower oil (13.1%), and high oleic sunflower oil (12.4%).*

[b] *Concentrations of phospholipids hereby indicated were values obtained in a previous production batch of similar infant formulas.*

[c] *Calculated concentrations of cholesterol were based on the cholesterol content of ingredients used in PL and D L(±Lf) formulas.*

Postmortem Sampling

According to our previous study on the kinetics of protein digestion, the 90-min postprandial sampling time is the optimal timepoint considering the progress of the digestion process and the appearance of dietary peptides in all digestive compartments from stomach to the ileum (16). Therefore, piglets were euthanized 90 min postprandially in the experimental slaughterhouse by electrical stunning immediately followed by exsanguination.

Blood was collected in tubes containing K^2-EDTA for glucose, insulin, haptoglobin, and lipid profiles and in tubes containing K^2-EDTA plus an anti–dipeptidyl peptidase-IV (DPP-IV, 10 μL/mL of blood) for GLP-1 (Millipore, Billerica, MA, USA). After centrifugation (10 min, 2,500 g, 4°C), plasma samples for glucose, insulin, haptoglobin, and lipid assays were stored at −20°C and the ones for GLP-1 assay at −80°C. Brain, liver, pancreas, perirenal, and subcutaneous adipose tissues were weighed. Duodenum, proximal and median jejunum, ileum, cecum, and colon were weighed (full and empty), and their length recorded. The digestive contents from the stomach, duodenum, proximal and median jejunum, ileum, and colon were collected by exerting a gentle pressure with the fingers.

Samples of digesta collected for *in vitro* intestinal secreting tumor cell line (STC-1) assays were stored at −20°C until used. Protease inhibitors were added in digesta collected for protein digestion analysis: pepstatin A (0.73 mM; 10 μL/mL of stomach digesta) and Pefabloc (0.1 M; 50 μL/mL of small and large intestinal digesta). Before storage at −20°C until further analysis, pH was measured. Digestive content aliquots were submitted to the direct lipid Folch extraction, as previously described (17). Briefly, 400 μL of digesta sample was mixed with 2.4 mL of chloroform/methanol (2:1 vol/vol) and acidified with 160 μL of HCl 0.1 N to stop lipolysis. The extract was then rinsed with NaCl 0.73% (100 μL) and 600 μL of chloroform/methanol (2:1 vol/vol). The chloroformic phase containing the lipid fraction was recovered and stored at −20°C for further lipid analyses. In addition, samples of the experimental formulas and digestive contents were collected for chemical and structural characterizations.

Proximal jejunal, ileal, and cecal tissue segments were rinsed with cold phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 48 h until further dehydration in ethanol and embedding in paraffin for morphometry analysis. Adjacent pieces of cecal and colonic tissues were rinsed with cold PBS and stored at −80°C until GLP-1 extraction and assay. Mucosa was scrapped from a 10-cm ileal segment for GLP-1 assays. One-cm^3 sample of pancreas was directly frozen and stored at −80°C for insulin extraction and analysis, and another cm^3 was fixed in 4% paraformaldehyde for immunohistochemistry.

Structural Characterization of Experimental Formulas and Digestive Contents

Particle Size Measurements

Particle size distribution was measured on the experimental formulas by laser light scattering using a Mastersizer 2000 (Malvern Instruments, Malvern, UK), with two laser sources at 466 and 633 nm. Refractive indexes used were 1.462 for vegetable oil and 1.333 for water (dispersion solution in the measurement cell). Samples were diluted in MilliQ-water in the measurement cell, either directly or after a 10-fold dilution in sodium dodecyl sulfate (SDS 1%), an anionic surfactant allowing aggregate dissociation. Measurements were performed in triplicates. Mean particle size distribution was represented by distribution curve, and mode diameter (diameter of the most frequent particles), volume weighted mean diameter (D[4,3]), and the specific surface area (SS) developed by the particles were calculated.

Confocal Laser Scanning Microscopy

The microstructures of the experimental formulas and of the digestive contents of all tissue segments of the intestine were observed as previously described in Bourlieu et al. (17). Briefly, a Nikon C1Si confocal laser scanning microscopy was used on an inverted microscope TE2000-E (Nikon, Champigny-sur-Marne, France) operated with an argon laser (excitation at 488 nm) and two He-Ne lasers (excitations at 543 and 633 nm). A 40× oil-immersion objective was used for all images, and three fluorescent dyes were used to localize apolar lipids (Lipidtox®), proteins (Fast Green), and amphiphilic compounds (Rhodamine-DOPE®). The microstructure was assessed on the three infant formulas and on the digesta from three pigs per formula group (one per replicate).

Biochemical Characterization

Proteins

SDS–polyacrylamide gel electrophoresis (PAGE) was performed on the experimental formulas and gastric contents using 4–12% polyacrylamide NuPAGE Novex *bis*-Tris 15-well-precast gels (Invitrogen, Carlsbad, CA, USA) and according to the manufacturer's instructions. Samples were diluted with NuPAGE® LDS sample buffer and then treated with 0.5 M DL-dithiothreitol and deionized water. The dilution of the formula by the gastric secretion was estimated based on the dry matter extract of the digesta compared to the dry matter of the formula. Twenty microliters of diluted sample containing 10 μg of protein was loaded into each well. Mark 12 Unstained Standard (Invitrogen) was used as a molecular weight (Mw) marker to identify the position of the bands. Gels were fixed in 40% (vol/vol) ethanol, 10% (vol/vol) acetic acid and 50% (vol/vol) deionized water and were rinsed for 30 min in deionized water before overnight staining with Bio-Safe Coomassie stain (Bio-Rad Laboratories, France). Discoloration of gels was performed with water. Image analysis of SDS-PAGE gels was carried out using Image Scanner III (GE Healthcare Europe GmbH, Velizy-Villacoublay, France), followed by densitometry of each band using the software Image Quant TL™ (GE Healthcare Europe GmbH, Velizy-Villacoublay, France). The percentage of each intact protein present in stomach 90 min postprandially was thus estimated in comparison with that present in the experimental formula.

NH_2 Quantification

The method was adapted from Darrouzet-Nardi et al. (18). It consisted in a spectrophotometric microplate analysis based on the reaction of orthophthaldialdehyde with primary amines in the presence of dithiothreitol resulting in 1-alkylthio-2-alkylisondole detected at 340 nm. The total content of primary amines in the experimental formulas was determined after total acid hydrolysis (6N hydrochloric acid, at 110°C for 24 h in vacuum-sealed glass tubes).

Peptidomic Analysis

Peptides in the three infant formulas and in the digestive samples (from stomach to colon, $n = 99$) were identified and quantified semiquantitatively by tandem mass spectrometry (Q-exactive, Thermo Scientific, Sans Jose, USA), such as described in Deglaire et al. (19). A home-made database for bovine milk proteins, based on the protein sequences as reviewed in uniport.org, was used for peptide identification. Bioactive peptides were searched within the database of BIOPEP (20), and prediction of antimicrobial activity was examined using CAMPR3 (21). Peptides were considered as antimicrobial peptide (AMP) when the score reached at least 0.5 on both methods that were support vector machine and random forest classifiers, recognized as the best-performing AMP predictors (22).

Lipid Analysis

Total Fatty Acids

The fatty acid composition of the formulas (C8:0–C24:0) was analyzed by gas chromatography coupled to a flame ionization detector by direct transmethylation, as described in Oliveira et al. (23). Samples were injected in duplicate.

Free Fatty Acids

Gastric free fatty acids (FFAs) (C4:0 to C20:0) were analyzed on gastric digesta by gas chromatography after lipid extraction using the Folch method and followed by a solid phase extraction (24). Three internal standards (160 μL of C5, C11, and C17 at 0.5 mg/mL) were added to samples prior to extraction as described previously (25). Samples were injected in duplicate. The gastric lipolysis degree was determined based on the total amount of FFA related to the estimated amount of total fatty acids present in the digestive content, the latter being determined based on the formula dilution such as indicated by the dry matter extract of the digesta.

Lipid Class Analysis

Thin-layer chromatography (TLC) was conducted to follow the evolution of the different lipid classes between stomach, proximal jejunum, and ileum digestive contents at 90 min postprandially as compared to undigested formula. A digesta volume equivalent to 60 μg of experimental formula lipids was spotted on silica gel plates (10 × 20 cm, 0.25 mm, Si G60, Merck). An Automatic TLC Sampler III (CAMAG, Muttenz, Switzerland) was used. Plates were immersed in hexane/diethyl ether/acetic acid (70:30:2

$v{\cdot}v^{-1}v^{-1}$) and then stained by immersion in copper sulfate II/orthophosphoric acid solution and heated (15 min, 150°C). Image analyses of the plates were performed as described for SDS-PAGE gels, allowing semiquantification of the different lipid classes, using the undigested formula as the reference.

Morphometry and Immunohistochemical Analyses

Seven-μm sections were stained with hematoxylin and eosin and examined under a light microscope (Nikon Eclipse E400, Nikon Instruments, France) using image analysis software (NIS-Elements AR 3.0, Nikon Instruments) as described (26). Villus, crypt, and goblet cell numbers were measured in at least 15–20 well-oriented crypt-villus units per piglet.

Fresh cecum, colon, and pancreas were fixed in 4% paraformaldehyde for 48 h at room temperature**.** They were then placed at 4°C in PBS containing 30% sucrose and embedded in the Tissue-Tek Optimum Cutting Temperature compound (Sakura Finetek Europe B. V., Zoeterwoude, the Netherlands), frozen in isopentane, and sectioned using a cryostat-microtome. Immunohistochemical analysis of cecum, colon, and pancreas was processed as previously described (11) to determine the number of enteroendocrine (chromogranin A-labeled) cells and GLP-1–secreting cells per area of mucosa, and the percentage of endocrine tissue and the number and diameter of islets in the pancreas.

Glucose, Lipid, Haptoglobin, Insulin, and GLP-1 Assays

Plasma glucose, FFA, triglycerides, total cholesterol, high-density lipoprotein cholesterol and haptoglobin were assessed by an automated spectrophotometric method (Konelab 20i, Thermo Fisher Scientific, Illkirsh, France) using specific commercial kits (Biomérieux, Bruz, France). The intra-assay coefficient of variation was <5%.

Insulin content was extracted from the pancreas by homogenization in 10 mL of an ethanol acid solution (75% absolute ethanol, 23.5% ultrapure H_2O, 1.5% HCl 12N) (Polytron 3100, Kinematica, 25,000 rpm, 2 × 20 s). After an overnight storage at −20°C, samples were centrifuged (30 min, 190 g, 4°C), and supernatants stored at −20°C. Ethanol acid solution (10 mL) was added to pellets for a second extraction, stored overnight at −20°C, and centrifuged (30 min, 190 g, 4°C). Supernatants were collected and pooled to the ones from the first extraction and diluted in a PBS/bovine serum albumin solution (1:3,000), and pancreas insulin concentration and plasma insulin were measured by a radioimmunoassay method, using iodinated porcine insulin as a tracer (INSULIN-CT, Cisbio International, Gif sur Yvette, France). The intra-assay CV and interassay CV were 15 and 11%, respectively, for a concentration of 35 μIU/mL.

GLP-1 content was extracted from ileal mucosa, cecum, and colon by homogenization of 1 g of tissue in 5 mL of ethanol acid solution (1% HCl 12M, 74% absolute ethanol, 25% H_2O) (Polytron 3100, Kinematica, 24,000 rpm, 2 × 20 s). After 24 h at 4°C, samples were centrifuged (20 min, 2,000g, 4°C), and supernatants diluted (1:1,000, 1:300, and 1:250 for ileal mucosa, cecum, and colon, respectively). Intestinal and plasma GLP-1 concentration was measured using a GLP-1 active enzyme-linked immunosorbent assay kit (Millipore), according to the manufacturer's instructions.

In vitro STC-1 Cell Assays

This assay aimed to evaluate *in vitro* the impact of jejunal and ileal contents on the GLP-1 secretion of STC-1 cells, a murine intestinal tumor cell line that possesses many features of native intestinal enteroendocrine cells. The intestinal STC-1 cell line was obtained from ATCC (ATCC®, CRL-3254™). The STC-1 cells were grown (37°C, 5% CO_2 atmosphere) in Dulbecco modified Eagle medium (4.5 g/L glucose) supplemented with 10% inactivated fetal calf serum (FCS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine. When reaching 80% confluence, cells were washed with PBS (pH 7.4), trypsinized, and seeded onto 24-well culture plates at a density of 40×10^3 cells per well and cultivated until they reached 70%−80% confluence. Cells were washed twice with media without FCS. Jejunal and ileal digesta were diluted (1:16 vol/vol) in incubation buffer (4.5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 140 mM NaCl, and 20 mM HEPES-Tris, pH 7.4) and centrifuged (10 min, 2,000 g, 4°C). Cells were then incubated for 2 h (37°C, 5% CO_2 atmosphere) with the digesta supernatants or with the incubation buffer (control wells). Finally, cell supernatants were collected, centrifuged (7 min, 2,000 g, 4°C), and kept at −20°C before GLP-1 radioimmunoassay measurement using an active GLP-1 kit (GLP1A-35HK, EMD Millipore, Billerica, MA, USA).

Statistical Analysis

Statistical analyses were performed using the R software, version 3.0.3 (27). Normality was tested with Shapiro and Wilk test. For parameters with unequal variances between groups, Box–Cox transformations were used. Differences between groups were assessed using a two-way analysis of variance (ANOVA) (lm function) testing formula composition, replication, sex, and interactions between formula and sex, and between formula and replication, followed by Tukey *post hoc* test (Tukey honestly significant difference). For lipid class characterization across intestinal sites, data were Box–Cox transformed and subjected to ANOVA for repeated measures (lme function) including formula, intestinal site, replication, sex, and interactions between formula and all other factors, followed by Tukey *post hoc* test (lsmeans package). Sex effect and the diet × sex interaction were not significant unless otherwise mentioned. Data are presented as mean values with their standard error of the mean (SEM). Differences were considered significant at $p < 0.05$ and a trend at $p < 0.1$. The graphical representation of the position and abundance of the peptides on the parent protein was performed using an in-house R script.

RESULTS

Effects of the Addition of Dairy Lipids and Probiotic Lf on Infant Formula and Digestive Content Structures

The three infant formulas were all characterized by a bimodal distribution of the particle size in water (**Figure 1A**, mode 1 =

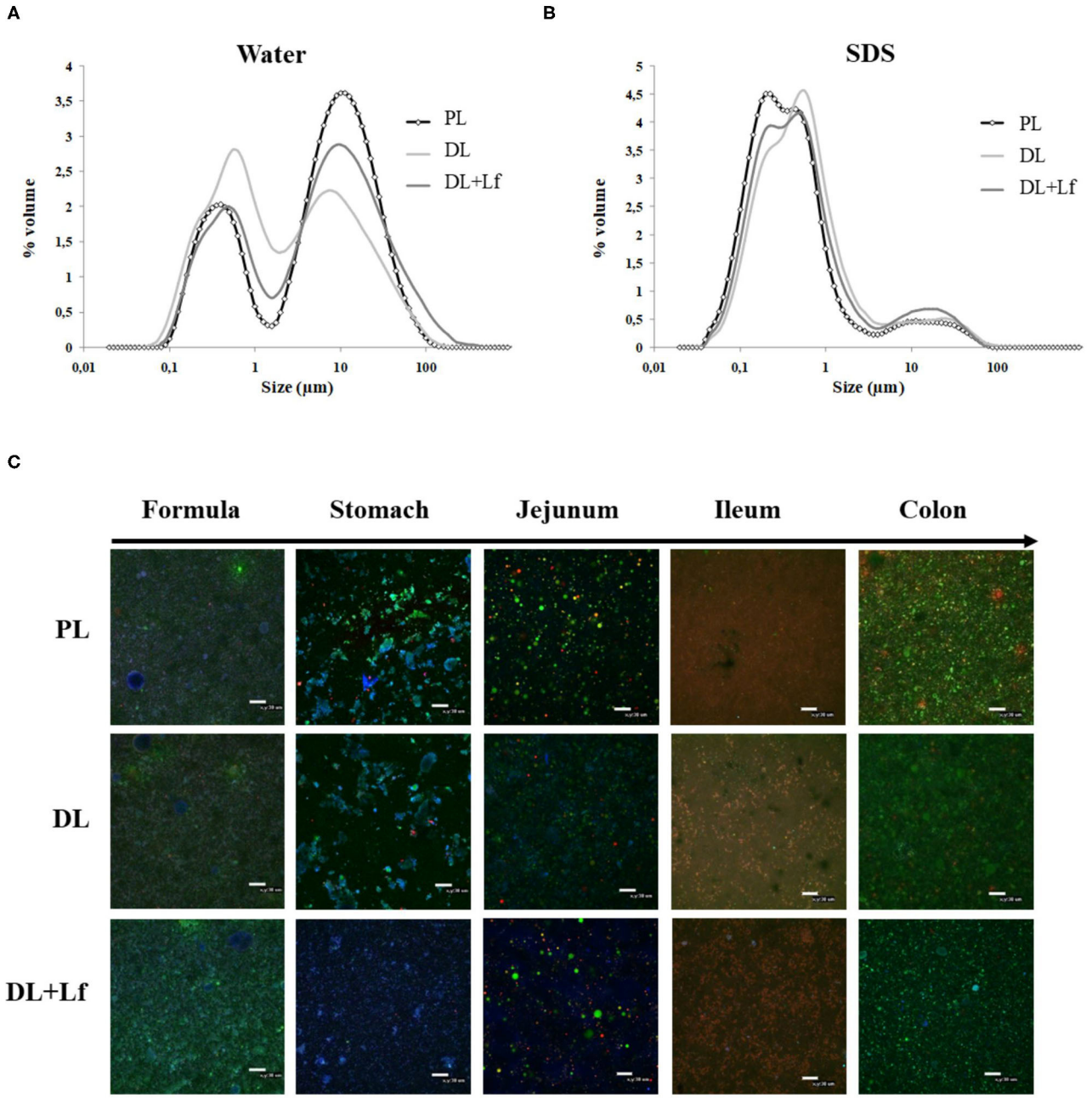

FIGURE 1 | Structural characterization of PL, DL, and DL+Lf formulas. **(A,B)** Particle size distribution of undigested PL, DL, and DL+Lf formulas determined by laser light scattering in **(A)** water and **(B)** SDS. Data represent the mean of three samples, each of them being measured in triplicate. **(C)** Confocal laser scanning microscopy images (objective ×40 zoom 1) of PL, DL, and DL+Lf undigested formulas and digestive contents from stomach to colon. Apolar lipids are colored in green (Lipidtox®), amphiphilic compounds in red (Rhodamine-PE®), and proteins in blue (fast green). Scale bars = 30 µm. Formulas contained as lipids either only plant lipids (PL), a half-half mixture of plant and dairy lipids (DL), or a half-half mixture of plant and dairy lipids supplemented with Lf (DL+Lf) sulfate.

9.77 µm and mode 2 = 0.52 µm). The biggest mode (mode 1) mostly disappeared after SDS addition (**Figure 1B**), indicating that these larger particles were aggregated droplets.

The microstructure of the digestive contents 90 min postprandially is given in **Figure 1C**. There was no major difference among formula groups within an intestinal site, whereas differences between intestinal sites were observed. The gastric phase was dominated by strong emulsion destabilization and protein aggregation. Lipid droplets were inserted within protein aggregates, which were likely formed due to the combined effect of acidic pH (3.4 in average at 90 min postprandially, **Table 2**) and proteolysis. The digesta

TABLE 2 | Digestive contents pH of PL, DL, and DL+Lf formulas.

pH	PL	DL	DL+Lf	Diet effect *p* value
Stomach	3.41 ± 0.30	3.37 ± 0.45	3.39 ± 0.30	0.98
Duodenum	4.64 ± 0.20	4.74 ± 0.30	4.80 ± 0.23	0.90
Proximal jejunum*	5.66 ± 0.15	5.42 ± 0.12	5.48 ± 0.13	0.17
Median jejunum	6.66 ± 0.10	6.90 ± 0.06	6.65 ± 0.14	0.20
Ileum	7.64 ± 0.11	7.72 ± 0.10	7.66 ± 0.15	0.46

*Data are expressed as the mean ± SEM. *Proximal jejunum: p(sex) < 0.01 (females > males).*
Formulas contained as lipids either only plant lipids (PL, n = 5–8), a half-half mixture of plant and dairy lipids (DL, n = 5–7), or a half-half mixture of plant and dairy lipids supplemented with Lf (DL+Lf, n = 4–7).

structure in jejunum greatly differed from that in the stomach, with fewer protein aggregates likely due to the digestive process (hydrolysis, emptying, and dilution by endogenous fluids) combined with the pH increase (**Table 2**). Mixed droplets of apolar and amphiphilic compounds, created by the bile salt emulsification, were also observed in the jejunum. There was mainly a colocalization of apolar lipids and amphiphilic molecules in the ileum (**Figure 1C**). The digesta structure in the colon was different from that of the ileum, with great quantity of apolar lipids, possibly originating from undigested lipids of the infant formula.

Gastric pH and intestinal pH (**Table 2**) were not impacted by the formula composition, increasing from the stomach up to the ileum.

Effects of the Addition of Dairy Lipids and Probiotic Lf on Piglet Growth, Body Composition, and Intestinal Morphology

BW gain, energy intake, and food efficiency were similar between groups during the lactation period (**Supplementary Figures 1A–C**). BW at euthanasia and tissue relative weights were similar between groups except for the liver (DL > PL), duodenum (tendency DL+Lf > DL), and the median jejunum (DL+Lf > PL and DL) (**Supplementary Table 1**). Total small intestinal length tended to be higher in DL+Lf compared with DL. Goblet cell number was higher in the proximal jejunum of DL+Lf than PL piglets, whereas villi surface tended to be lower in the ileum of DL+Lf than PL piglets (**Supplementary Table 1**); neither was different to DL.

Effects of the Addition of Dairy Lipids and Probiotic Lf on Proteolysis

Gastric protein digestion was followed by SDS-PAGE (**Figure 2A**). Regardless of the formula, casein was more extensively digested than β-lactoglobulin and α-lactalbumin 90 min postprandially (**Figure 2A**). The percentage of intact casein was lower in DL+Lf stomach compared with PL and DL, highlighting a decreased resistance of this protein to gastric digestion in the presence of DLs and probiotic Lf (**Figure 2A**). Intact protein percentages of β-lactoglobulin and α-lactalbumin were similar between groups. The overall gastric protein hydrolysis was higher in DL and DL+Lf piglets than in PL piglets (**Figure 2B**).

Overall, before and during digestion, 2,758 unique peptides (6–46 amino acids in length) were identified and derived from 19 parent proteins, of whom 8 were caseins (**Supplementary Table 2**). Forty to sixty peptides, mainly derived from caseins, were identified before digestion in the three infant formulas.

Of the 2,758 identified peptides, 1,954 peptides (still originating from the aforementioned 19 parent proteins, **Figure 3**) were kept for further analyses because they were present in at least half (or half+1 in case of odd number) of the digesta samples of one of the three groups at a given site. Peptide diversity was the highest in the stomach and in proximal jejunum and largely decreased in the lowest part of the intestine (**Figure 3**). A large percentage of peptides were common between consecutive digestive sites, especially between proximal and the median jejunum (**Figure 3**). Throughout digestive sites, peptides mainly originated from β-casein (CSN2A2) and β-lactoglobulin (BLGA) (**Figure 3**). Distribution of peptides along these parent protein sequences was similar between groups in the stomach until the median jejunum but was more different in the ileum and the colon (**Figure 4**). The average peptide abundance of β-lactoglobulin and β-casein was the highest for PL and the lowest for DL+Lf from stomach to median jejunum, whereas a higher peptide abundance was observed in ileum for DL and in colon for DL+Lf especially on β-casein (**Figure 4**).

The number of identified bioactive peptides and AMPs was the highest in the stomach (**Figure 3**). Some bioactive peptides and potential AMPs were still found in proximal jejunum, but a reduced number (maximum of 4) were observed from median jejunum to the colon. Bioactive peptides originated from β-lactoglobulin, β-casein, α-s1 and α-s2 casein, and κ-casein in the stomach; from β-lactoglobulin, β-casein, α-s1 and α-s2 casein, and α-lactalbumin in the proximal jejunum; and from β-casein in the median jejunum, ileum, and colon. One peptide originated from α-s1 casein in DL+Lf ileum. Throughout the digestive sites, identified bioactivities were antibacterial, immunomodulating, dipeptidyl peptidase IV inhibitor, angiotensin-converting enzyme inhibitor, antiamnestic, binding, hamolytic, anticancer, opioid agonist, and antioxidative. Predicted AMPs originated from β-lactoglobulin, α-s1 and α-s2 casein, κ-casein, xanthine dehydrogenase/oxidase, lactoperoxidase, osteopontin, and serum albumin in the stomach; β-lactoglobulin and β-casein in proximal jejunum; and β-lactoglobulin in median jejunum, ileum, and colon. Bioactive peptides and predicted AMPs originating from the two major proteins, β-lactoglobulin and β-casein, are presented in **Figure 4**; for clarity, gastric AMPs are not shown.

Effects of the Addition of Dairy Lipids and Probiotic Lf on Lipolysis

Fatty acid composition of the infant formulas is given in **Figure 5**. Except for *trans*-fatty acid content, the three infant

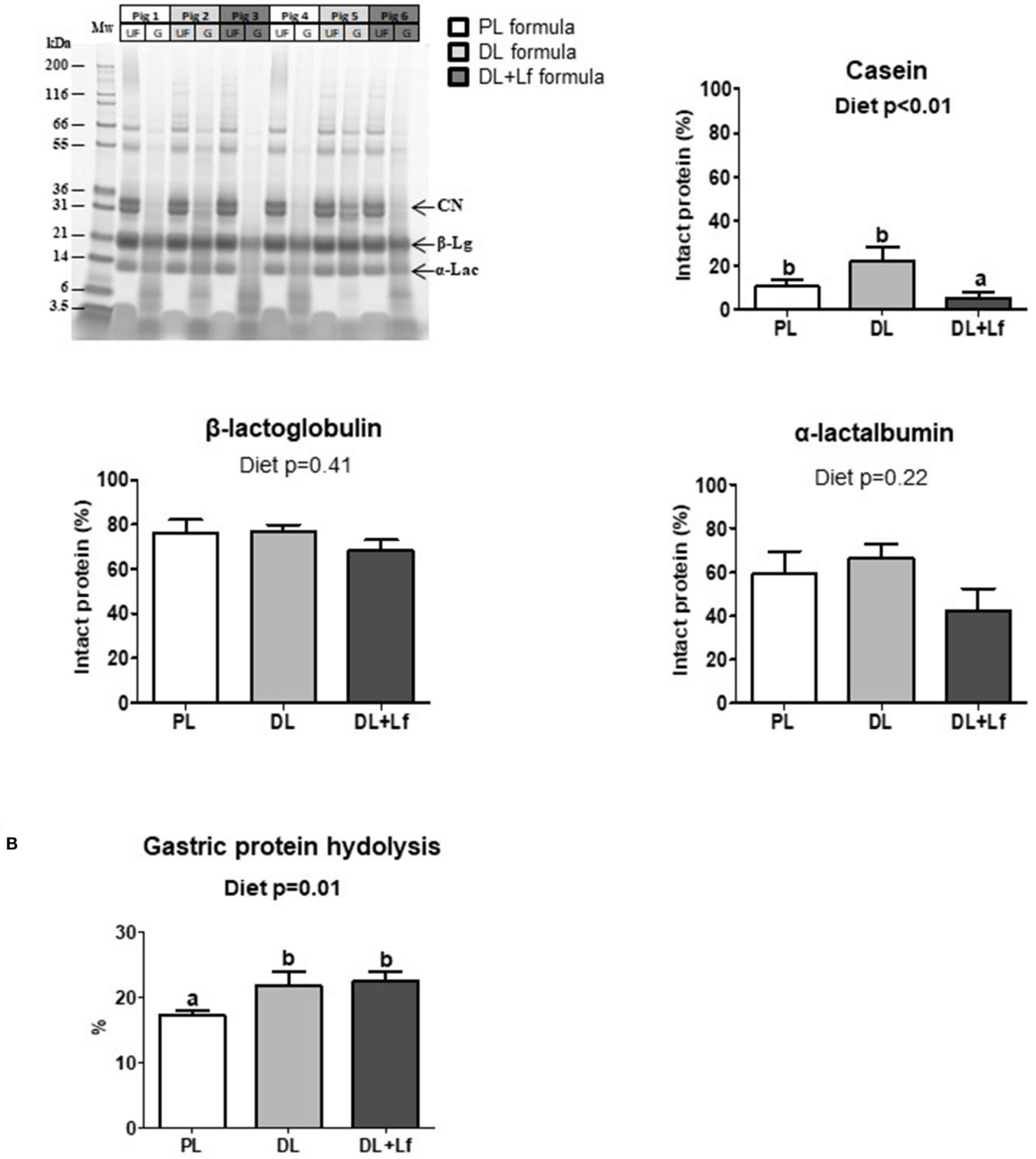

FIGURE 2 | Gastric proteolysis of PL, DL, and DL+Lf formulas. **(A)** Example of SDS-PAGE protein profiles of PL, DL, and DL+Lf formulas during gastric *in vivo* digestion. Protein molecular mass standards (Mw) are on the left followed by the undigested infant formula (UF) and corresponding gastric content (G) for the same pig (side by side). Gastric proteolysis resistance: corresponding percentage of intact proteins [casein (CN), β-lactoglobulin (β-Lg), and α-lactalbumin (α-Lac)] during gastric digestion, estimated by densitometry in comparison to the corresponding undigested formula. Data are expressed as the mean ± SEM. Labeled means without a common letter differ ($p < 0.05$). **(B)** Gastric proteolysis evaluated by NH2 quantification. Formulas contained as lipids either only plant lipids (PL, $n = 7$–8), a half-half mixture of plant and dairy lipids (DL, $n = 6$–8), or a half-half mixture of plant and dairy lipids supplemented with Lf (DL+Lf, $n = 6$). SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CN, casein; β-Lg, β-lactoglobulin; α-Lac, α-lactalbumin.

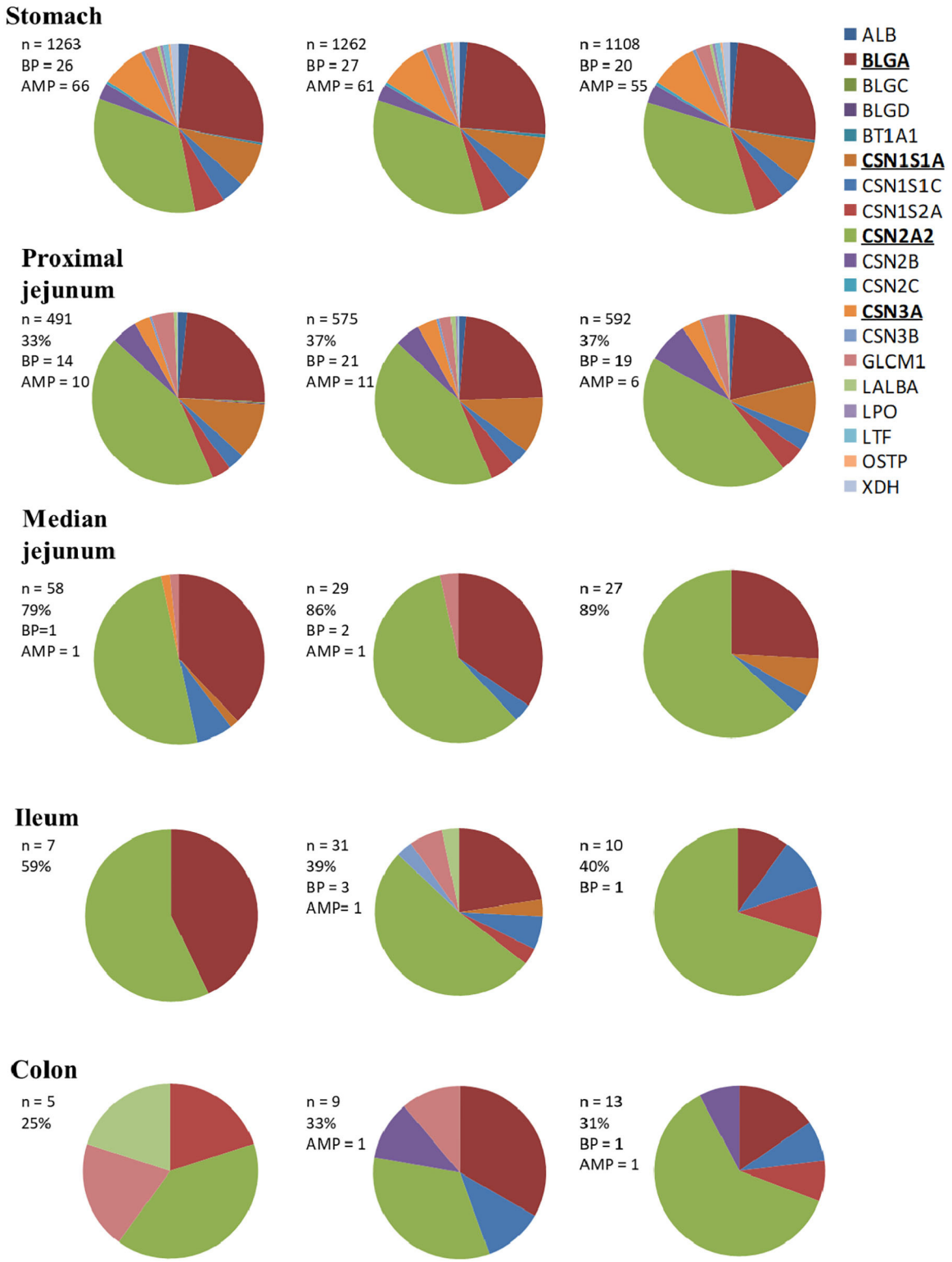

FIGURE 3 | Pie chart distributions of peptides by parent proteins throughout the gastrointestinal tract. Proteins that appear in bold and underlined are the main parent proteins. In the insert, the following information are given: n, number of peptides; %, % of peptides common with the preceding compartment; BP, bioactive peptides; AMP, predicted anti-microbial peptides. Formulas contained as lipids either only plant lipids (PL, $n = 5$–8), a half-half mixture of plant and dairy lipids (DL, $n = 5$–8), or

(Continued)

FIGURE 3 | a half-half mixture of plant and dairy lipids supplemented with Lf (DL+Lf, $n = 6–7$). ALB, serum albumin; BLGA, β-lactoglobulin A; BLGC, β-lactoglobulin C; BLGD, β-lactoglobulin D; BT1A1, butyrophilin; CSN1S1A, casein α-S1 A; CSN1S1C, casein α-S1 C; CSN1S2A, casein α-S2 A; CSN2A2, β-casein A2; CSN2B, β-casein B; CSN2C, β-casein C; CSN3A, κ-casein A; CSN3B, κ-casein B; GLCM1, glycosylation-dependent cell adhesion molecule; LALBA, α-lactalbumin; LPO, lactoperoxydase; LTF, lactoferrin; OSTP, osteopontin; XDH, xanthine dehydrogenase/oxidase.

formulas had similar content of the different classes of fatty acids (saturated and unsaturated fatty acids). However, the main individual fatty acids greatly differed among formulas, with higher levels of C18:1 and C16:0 and lower levels of C18:0 and C14:0 in PL than in DL and DL+Lf. As expected, the PL formula containing only vegetable lipids merely contains fatty acids ranging from C6:0 to C10:0, also called medium-chain fatty acids (MCFAs), and DL and DL+Lf formulas incorporating DLs as cream contained much more of these MCFAs.

Gastric lipolysis degree was very low (<1%) and similar for all three groups. Along the digestive tract, lipolysis increased from the stomach to the ileum 90 min postprandially and was characterized by a strong decrease in triacylglycerols and an increase in lipolysis products (FFAs and diacylglycerols and monoacylglycerols) (**Figure 6A**). The kinetics of appearance of diacylglycerols/cholesterol were significantly impacted by the infant formula composition, depending on the intestinal site (**Figure 6B**). A higher percentage of diacylglycerols/cholesterol was observed in DL stomach and in DL and DL+Lf proximal jejunum and ileum compared with PL. Regardless of formula, this percentage was increased in the proximal jejunum compared with the stomach and decreased in the ileum compared with the proximal jejunum. The kinetics of appearance of FFAs and monoacylglycerols/polar lipids in small intestinal contents were not significantly different amongst formulas (data not shown). The triacylglycerols:total lipid ratio in proximal jejunum was similar between groups (**Figure 6C**). In contrast, it tended to decrease in DL+Lf ileum compared with PL (**Figure 6D**), reflecting an increased lipolysis.

Effects of the Addition of Dairy Lipids and Probiotic Lf on Metabolism and Entero-Insular Axis

The infant formula composition barely influenced the plasma lipid profile with tendencies to decrease plasma FFA and triglyceride concentrations in DL and DL+Lf piglets compared with PL (**Figure 7A**) and did not influence plasma glucose, insulin, and insulin:glucose ratio, and GLP-1 and haptoglobin concentrations (**Figures 7B–D**). The percentage of endocrine tissue and the number of Langerhans islets in the pancreas were decreased in DL+Lf compared with DL with no effect on the pancreas insulin content (**Table 3**). Mean islet diameter tended to be increased in DL compared with PL and DL+Lf (**Table 3**) without any difference in the pancreatic size islet distribution (data not shown). However, the infant formula composition had no effect on GLP-1 concentration and GLP-1-secreting cell number in the ileum, cecum, and colon (**Supplementary Table 3**). Finally, the capacity of jejunal and ileal contents to induce GLP-1 secretion by STC-1 cells did not significantly differ between formulas (**Figure 8**).

DISCUSSION

To our knowledge, this is the first study evaluating the impact of the addition of DLs with or without probiotic Lf in infant formulas on digestion, gut physiology, and metabolism. This study demonstrated, through the use of a set of multiscale techniques, that the addition of DLs and probiotic Lf in infant formulas affected protein and lipid digestion, decreased the endocrine tissue in the pancreas, and increased the intestine weight and the jejunal goblet cell density in Yucatan minipiglets.

A classical structure of homogenized infant formulas was observed with submicronic fat droplets in all the formulas, although most of them were in an aggregated form in the three formulas, as observed after the addition of an anionic surfactant. The observed microstructure in digesta differed alongside the digestive tract, but was not different among formulas with the present magnification. Different interfacial composition between DLs and PLs may have been present; however, interface characterization was not performed. This could be achieved after extracting and washing the lipid droplets, followed by chromatography characterization of proteins and phospholipids at the interface.

The infant formula composition affected the overall gastric proteolysis that was increased in the two groups that received DLs, potentially resulting from different interfacial composition between dairy and plant lipids. This result was in line with the lower concentration of residual intact caseins in DL+Lf stomach, but not with that in DL stomach. The former observation could be partly induced by a faster gastric emptying, as the fraction of the ingested meal remaining in stomach 90 min postprandially was the lowest in DL+Lf compared to DL and PL (24 vs. 33 and 36%, respectively), although this did not reach statistical significance. The values obtained for DL and PL were in agreement with previous results indicating that 39% of total ingested nitrogen remained in the stomach 90 min postprandially, vs. 79% 30 min after meal ingestion (16). It is unlikely that the increased proteolysis was related to a proteolytic effect of the probiotic Lf since Cardenas et al. (28) did not reveal a remarkable proteolytic activity of probiotic Lf.

Caseins, particularly β-casein, were identified in our study as the main parent protein of the peptides present in the three experimental formulas and in digestive contents. Differences in protein digestion and casein resistance previously discussed may therefore affect the release of peptides and protein/peptide biological activities. In agreement with the decreased intact casein in the DL+Lf stomach, peptides belonging to β-casein were less abundant in the stomach and proximal and median jejunum but more abundant in the colon of DL+Lf than in that of PL and DL. This result is in accordance with a faster gastric emptying induced by dairy lipids and probiotic Lf. Different bioactive peptides were observed along the sequence of β-casein

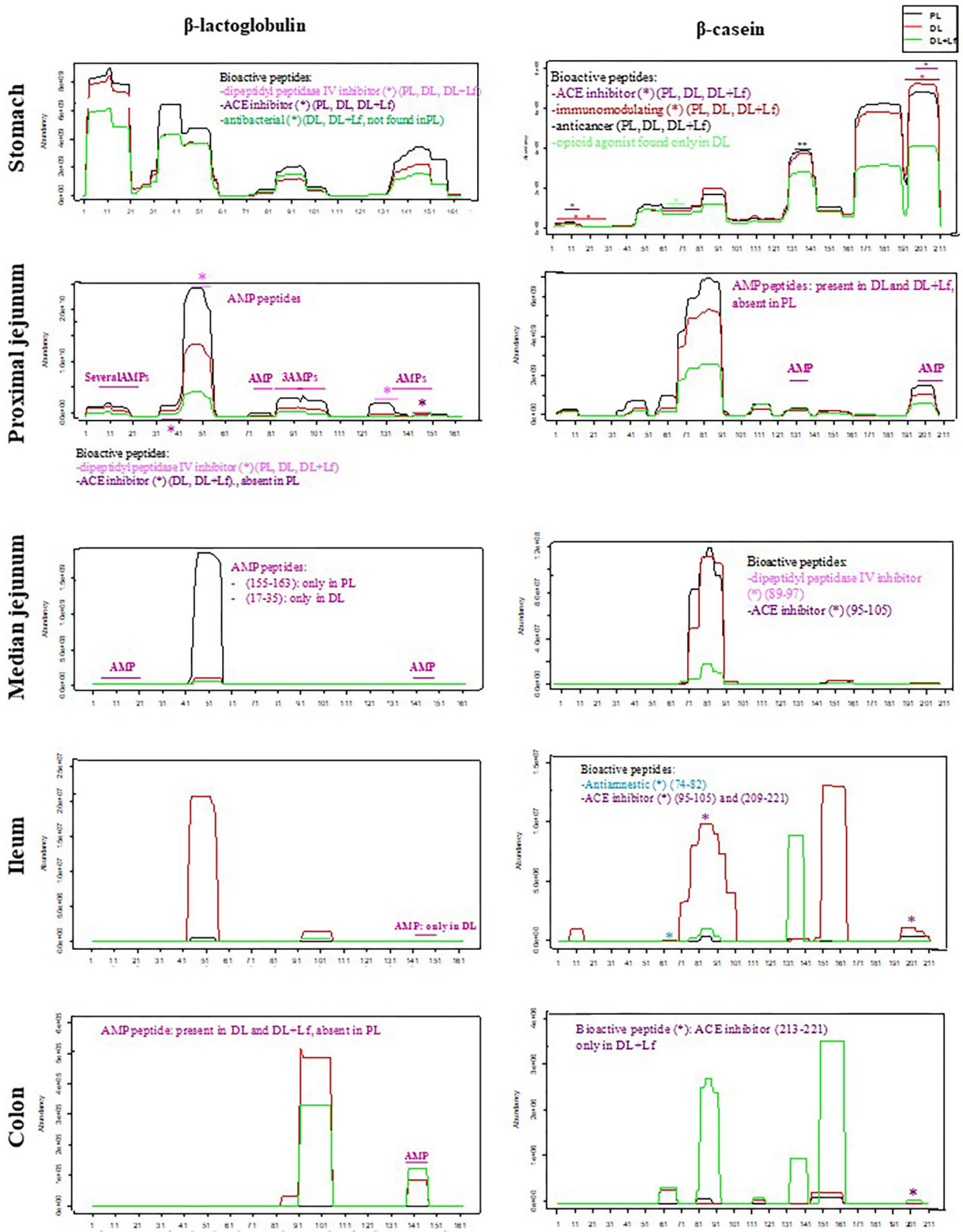

FIGURE 4 | Abundance of peptides belonging to β-lactoglobulin (BLGA) and β-casein (CSN2A2) proteins and identification of bioactive peptides and AMPs throughout the gastrointestinal tract. Bioactive peptides and AMPs are identified by a star colored according to their predicted bioactivity. Formulas contained as lipids either only plant lipids (PL, $n = 5–8$), a half-half mixture of plant and dairy lipids (DL, $n = 5–8$), or a half-half mixture of plant and dairy lipids supplemented with Lf (DL+Lf, $n = 6–7$). A * indicates bioactive peptide or AMP peptides. AMP, antimicrobial peptide; ACE, angiotensin-converting enzyme.

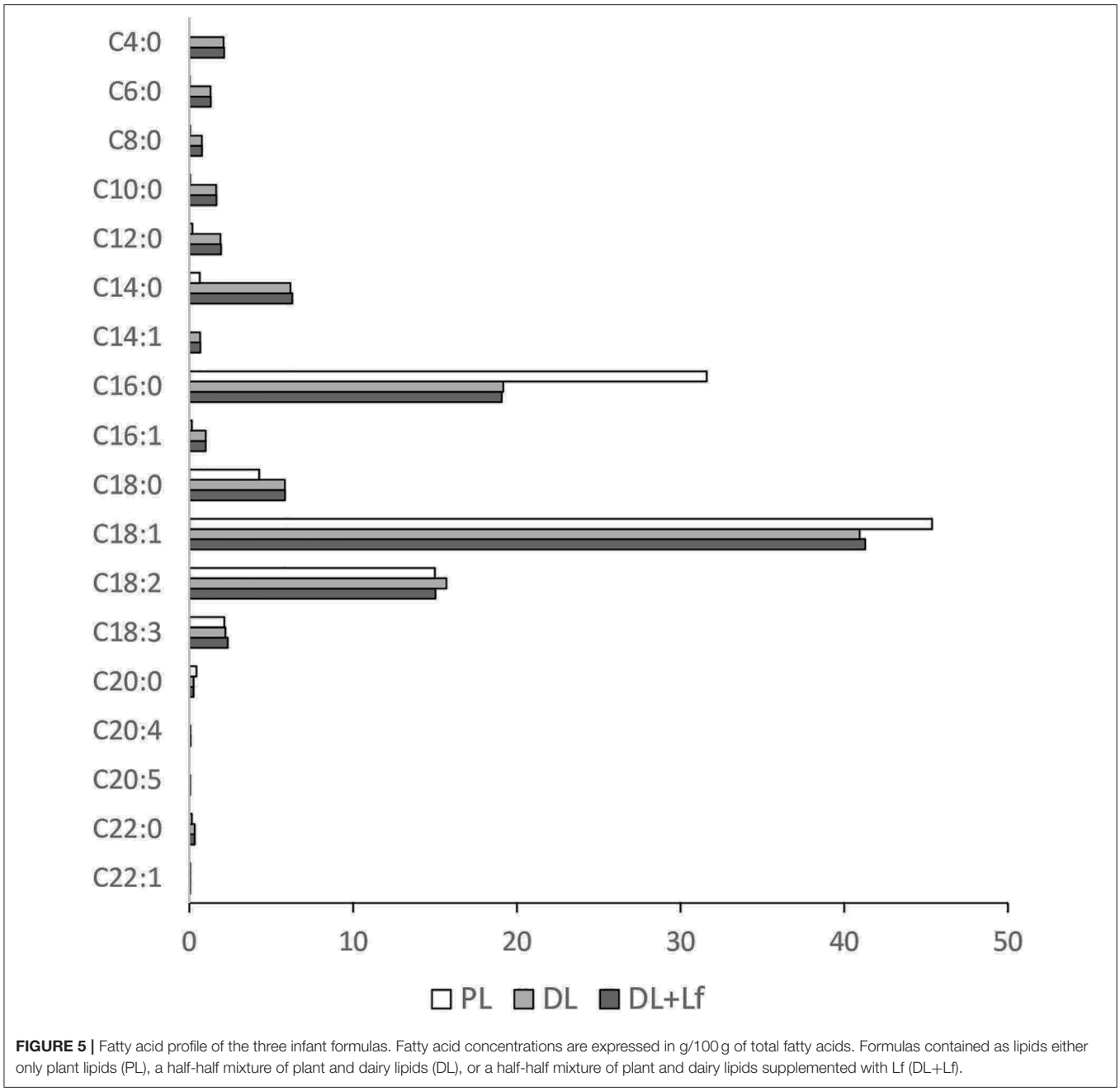

FIGURE 5 | Fatty acid profile of the three infant formulas. Fatty acid concentrations are expressed in g/100 g of total fatty acids. Formulas contained as lipids either only plant lipids (PL), a half-half mixture of plant and dairy lipids (DL), or a half-half mixture of plant and dairy lipids supplemented with Lf (DL+Lf).

and β-lactoglobulin. Particularly, an inhibitor of the DPP-IV was found to be less abundant for DL + Lf than for the other formulas from the stomach until the median jejunum. The DDP-IV is produced all along the intestine, and significant DPP-IV–like activity occurs in the microbiota (29). This peptidase is involved in the last step of dietary protein digestion, with the specificity of being able to hydrolyze peptides containing proline, unlike pancreatic proteases. Thus, the reduction of DPP-IV activity may alter protein digestion and absorption (29). Besides, DPP-IV is known to inactivate two incretins, GLP-1 and GIP, involved in the control of glucose metabolism. In the present study, such effect was not apparent as plasma GLP-1 contents were similar among groups. This can be explained by the fact that the DPP-IV activity in the bloodstream is mainly that of hematopoietic and endothelial cells.

Different distributions of peptide origin were observed between groups from median jejunum to colon. Especially, PL had lower peptide diversity in the ileum and the colon compared to DL and DL+Lf, highlighting a specific effect of dairy lipids on proteolysis. These different peptides may differentially modulate gut microbiota and gut physiology, or on the other hand, dairy lipids and probiotic Lf may have differentially modulated gut

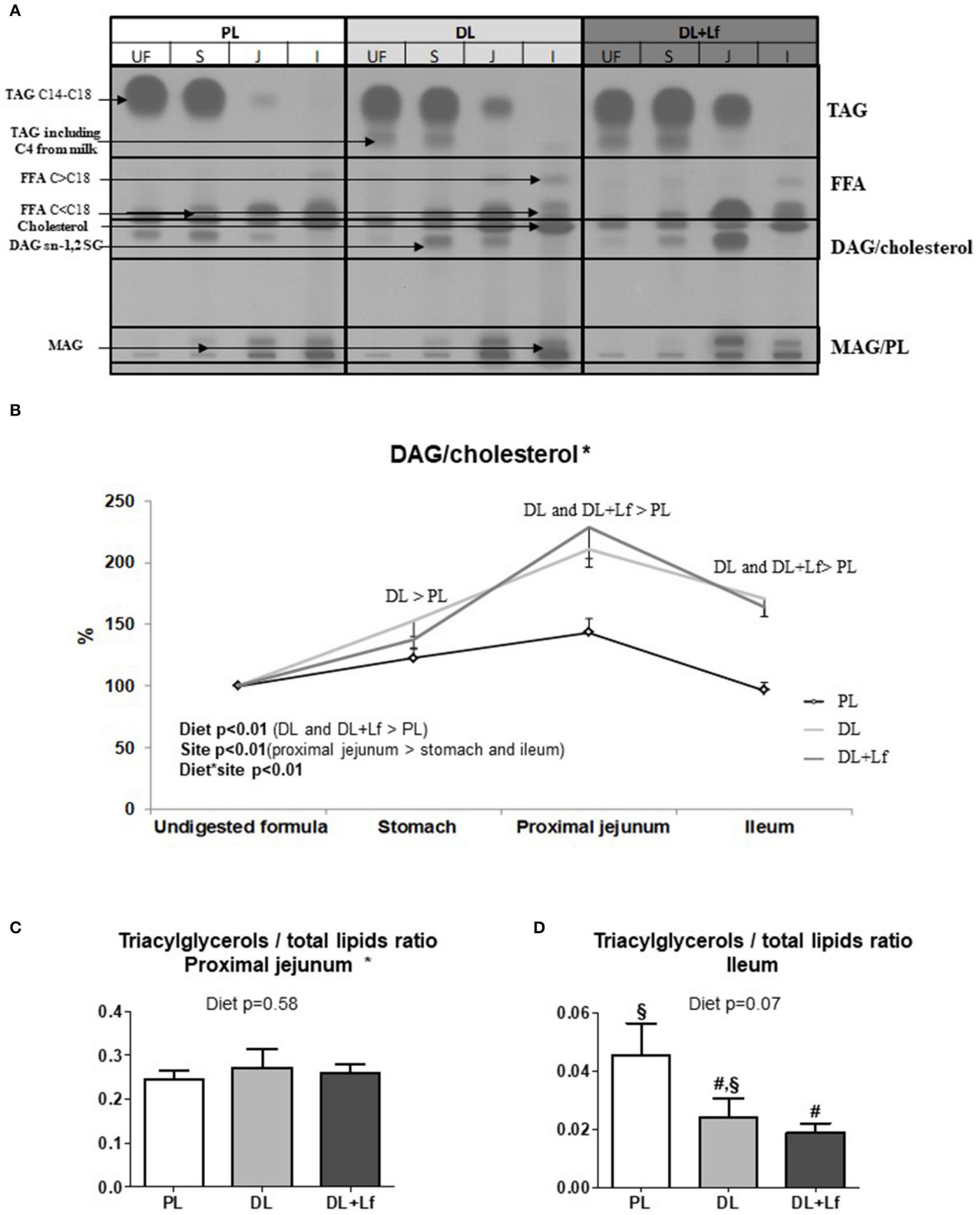

FIGURE 6 | Gastric and intestinal lipolysis of PL, DL, and DL+Lf formulas. **(A)** Example of thin-layer chromatography showing the evolution of lipid classes between stomach (S), proximal jejunum (J), and ileum (I) 90 min postprandially compared to undigested formula (UF). **(B)** Longitudinal evolution of DAG/cholesterol along the intestine based on the undigested infant formula content. All written effects were significant ($p < 0.01$). **(C)** Triacylglycerols/total lipids ratio in proximal jejunum and

(Continued)

FIGURE 6 | (D) in ileum. Labeled bar charts without a common symbol differ ($p < 0.1$). Formulas contained as lipids either only plant lipids (PL, $n = 7–8$), a half-half mixture of plant and dairy lipids (DL, $n = 6–8$), or a half-half mixture of plant and dairy lipids supplemented with Lf (DL+Lf, $n = 6–7$). TAG, triacylglycerol; FFA, free fatty acids; SC, short chain; DAG, diacylglycerol; MAG, monoacylglycerol; PL, polar lipid. Data are expressed as the mean ± SEM. *DAG/cholesterol: p(diet * sex) = 0.04 (DL and DL+Lf females > PL females, DL, and DL+Lf male piglets > PL male piglets); TAG/total lipids ratio in proximal jejunum: p(diet * sex) = 0.02 (DL males > DL females) and p(sex) is not significant.

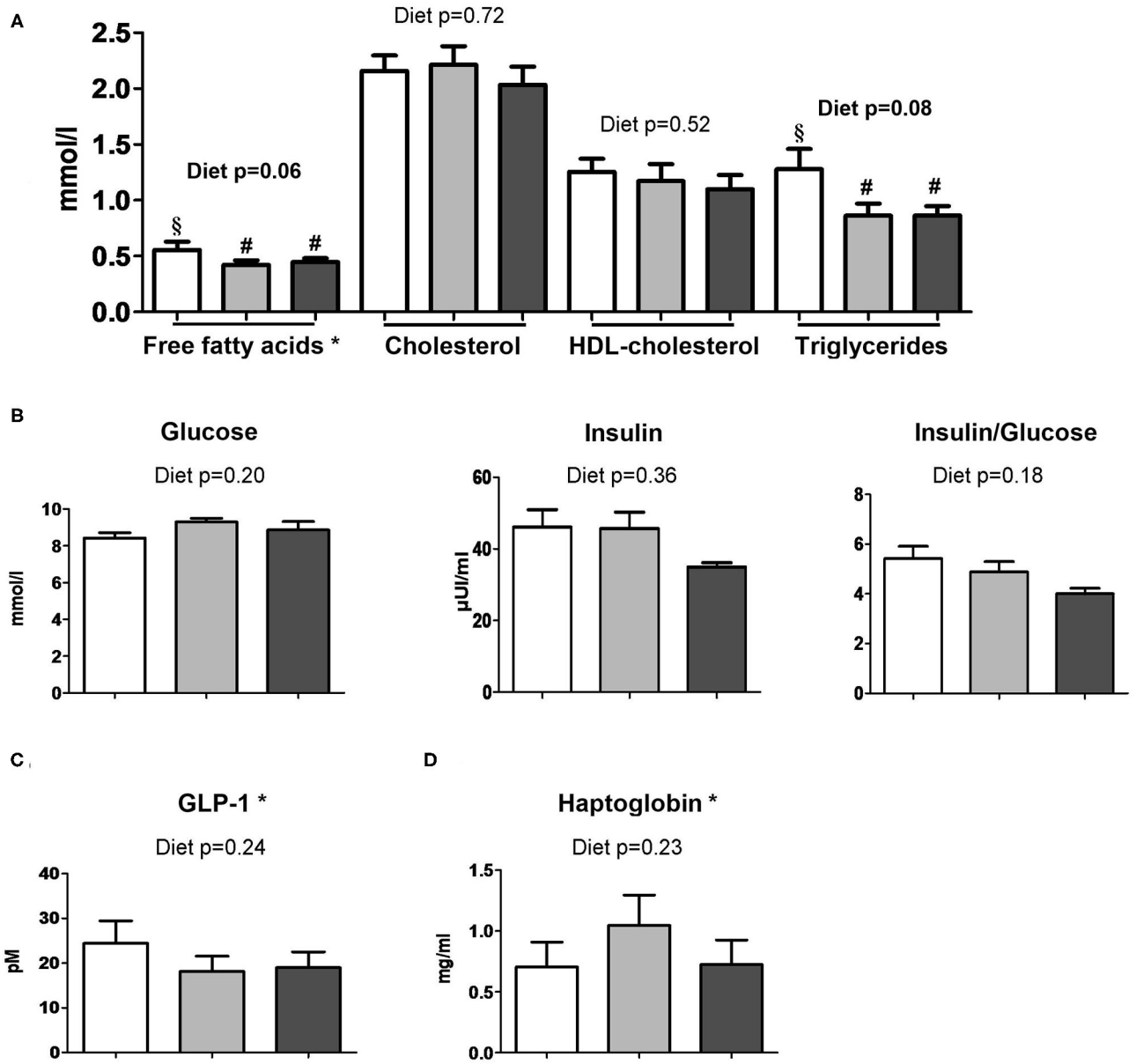

FIGURE 7 | Plasma metabolic profiles of PL, DL, and DL+Lf piglets. Plasma **(A)** lipid profile, **(B)** glucose, insulin and insulin:glucose ratio, **(C)** GLP-1, and **(D)** haptoglobin concentrations 90 min postprandially. Formulas contained as lipids either only plant lipids (PL, $n = 6–8$), a half-half mixture of plant and dairy lipids (DL, 6–7), or a half-half mixture of plant and dairy lipids supplemented with Lf (DL+Lf, $n = 6–7$). Data are expressed as the mean ± SEM. Labeled means between bar graphs without a common letter or symbol differ ($p < 0.05$ and $p < 0.1$, respectively). HDL, high-density lipoprotein; GLP-1, glucagon-like peptide-1. *Free fatty acids: p(diet * sex) = 0.03 (PL females > DL and DL+Lf females) and p(sex) = 0.82; GLP-1: p(sex) = 0.02 (females > males); haptoglobin: p(diet * sex) = 0.05 (DL females > PL females) and p(sex) = 0.45.

physiology and consequently its digestive capacity. Particularly, the present study has demonstrated a beneficial effect of probiotic Lf on non-specific host defenses, increasing goblet cell density in jejunum, in agreement with Lf-induced greater MUC-2 expression observed *in vitro* in HT29 cells (30). It should be noted that the intestinal transit time may also have been changed

TABLE 3 | Endocrine pancreas parameters of PL, DL, and DL+Lf piglets.

Endocrine pancreas	PL	DL	DL+Lf	Diet effect *p* value
Endocrine tissue (%)*	1.79 ± 0.17^{a,b}	2.28 ± 0.24^{b}	1.58 ± 0.07^{a}	0.02
No. of islets (per 0.5-cm^2 tissue)	250 ± 21^{a,b}	291 ± 22 ^{b}	221 ± 9^{a}	0.05
Mean islet diameter (μm)*	54.6 ± 0.9$^{\#}$	56.7 ± 1.2§	54.7 ± 0.7$^{\#}$	0.09
Insulin content (IU/g of pancreas)	14.3 ± 0.5	15.3 ± 1.3	13.7 ± 1.0	0.58

*Data are expressed as the mean ± SEM. Labeled means in a row without a common letter or symbol differ ($p < 0.05$ and $p < 0.1$, respectively). *Endocrine tissue: p(sex) = 0.1(males > females); endocrine pancreas mean islet diameter: p(sex) < 0.01 (males > females). Formulas contained as lipids either only plant lipids (PL, n = 6–8), a half-half mixture of plant and dairy lipids (DL, n = 6), or a half-half mixture of plant and dairy lipids supplemented with Lf (DL+Lf, n = 6–7).*

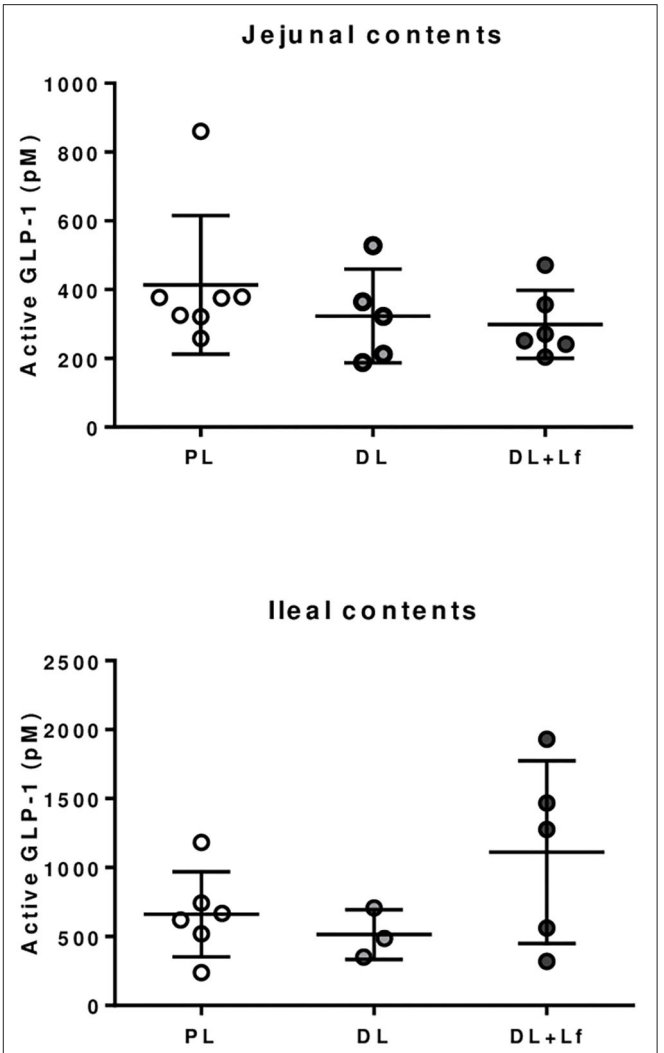

FIGURE 8 | GLP-1 secretion by STC-1 endocrine cells in presence of jejunal and ileal contents of PL, DL, and DL+Lf piglets. Formulas contained as lipids either only plant lipids (PL, *n* = 6–7), a half-half mixture of plant and dairy lipids (DL, *n* = 3–5), or a half-half mixture of plant and dairy lipids supplemented with Lf (DL+Lf, *n* = 5–6).

by specific peptides released during casein digestion such as the opioid ones (31, 32).

Another important result was that the infant formula composition modulated lipid digestion. This was not apparent at the stomach level, where lipolysis appeared to be low; however, this may have been underestimated because of the gastric emptying of the FFAs released before digesta collection. The percentage of diacylglycerols/cholesterol present at 90 min postprandially was higher for DL and DL+Lf than for PL in proximal jejunum and ileum, which is likely due to a higher abundance of diacylglycerols sn-1,2 from DL and DL+Lf compared with PL. Concomitantly, tendencies to decreased plasma FFA and triglyceride concentrations in DL and DL+Lf piglets compared with PL were observed. This could be the result of a different metabolic fate of the lipolysis products and/or a reduced and/or slower lipid absorption with dairy lipids. Differences observed among groups in terms of lipolysis may therefore trigger health effect. In addition, the nature of the fatty acid released can also play different luminal and systemic functions. Our previous study on milk fat and MFGM fragments in piglets showed a significant increase of the mucosal immune system maturation and modification of the fecal microbiota composition (8). Particularly, the sphingosine and MCFAs present in dairy lipids are known for their antimicrobial activity and their modulation of gut microbiota establishment with increased *Lactobacillus* and *Bifidobacterium* (33). Contrary to what was expected, no difference in plasma cholesterol was observed between groups. It is noteworthy that the cholesterol content of the experimental formulas containing dairy lipids was lower than that of sow milk [0.80 and 10.7 mg for 100 mL of PL and DL (±Lf) formulas, respectively, vs. 145 mg for 100 mL of sow milk (34)].

Overall metabolism was not affected by the infant formula composition. However, our results highlighted that probiotic Lf decreased the percentage of endocrine pancreas and the number of Langerhans islets. Similar effect of "pancreatic savings" was observed in piglets supplemented with prebiotic short chain fructooligosaccharides (scFOS), suggesting a lower insulin demand and potentially a better insulin sensitivity of peripheral tissues to insulin (35) even if the postprandial insulin and the insulin/glucose ratio did not display a significant decrease in DL+Lf. As supplementation of both probiotic Lf and prebiotic scFOS is susceptible to modify intestinal microbiota, this "pancreatic savings" could be related to intestinal microbiota modification between groups. Intestinal GLP-1 secreting function (i.e., number and percentage of GLP-1 secreting enteroendocrine L-cells) and postprandial GLP-1 concentration were not different between groups. This was in agreement with the *in vitro* STC-1 results demonstrating no difference in the GLP-1 secretion stimulating capacity of

intestinal contents, despite differences in their peptide and lipid compositions. In contrast, we reported a long-term promoting effect of Lf on GLP-1 secreting function in the adult minipigs under a hyperenergetic-diet challenge, suggesting that a deleterious nutritional environment was necessary to reveal the metabolic programming (11). Finally, a trophic effect of probiotic Lf was observed on duodenum and jejunum weights, as well as on small intestine length. This is coherent with a recent study that also observed an intestinal trophic effect of this specific strain (36). Mechanisms responsible for the modulation of intestinal growth by Lf remain unknown, but could involve, for instance, a modulation of microbiota composition (11).

The use of piglets as models for infants was a strength of our study, the piglet having many common features with infant regarding nutritional physiology and functional gut maturation (7). Furthermore, regarding lipid digestion, as for breast milk, palmitic acid in sow milk is mainly on the sn-2 position 70%, (37), and as for humans, pig pancreatic lipase mainly hydrolyzes fatty acids on the sn-1,3 positions, and palmitic acid is well-absorbed through the gut epithelium as a monoacyl glycerol (38). Therefore, piglets are very good models for infants in this regard. However, our study also displayed some limitations. For instance, it would have been optimal to have a fourth group of animals receiving the PL formula plus probiotic. However, because of economical and ethical constraints, we had to limit the number of experimental piglets and could consider only three groups. In particular, we selected the three groups PL, DL, and DL+Lf to achieve our goal of investigating the role of re-introduction of dairy lipids in infant formula on its digestion and on the intestinal physiology, and its eventual synergy with probiotic Lf. Also, a basal (fasting) point would be needed to evaluate the amplitude of the postprandial (90 min) response and compare it between formulas. However, as infants, piglets eat every 2 h during the suckling period, so there is technically no fasting period that could have been taken as a reference. The timepoint 90 min was therefore a good compromise for assessing the digestion process in suckled minipigs (16).

In conclusion, our data provide interesting knowledge about how infant formulas with different lipid nature (plant vs. dairy) and probiotic content may be differently digested, with consequences on gut physiology. More precisely, the addition of dairy lipids in infant formula modulated the digestion of lipids, whereas the addition of Lf increased proteolysis, had an intestinal trophic effect, increased the number of goblet cells, and induced a "pancreas savings" effect. Our results corroborate the synergistic properties of MFGMs and probiotics reported on mucosal B- and T-cell proliferation and mucosal IgA-secreting cells (39). The adhesion of lactic acid bacteria strains to MFGM, previously demonstrated for *Lactobacillus reuteri*, may participate in the greater impact of the combination of dairy lipids and Lf compared to individual ingredients (40). All these effects had potential nutritional relevance due to bioactive peptides and AMPs and lipolysis products, and displayed beneficial, although moderate, effects on non-specific host defenses and intestinal size, as well as metabolism. Overall, we did not notice any effect of dairy lipids with or without Lf on piglet growth. This was also the case in infants receiving formulas supplemented with either MFGM or another probiotic strain (*Lactobacillus paracasei* ssp. *paracasei* strain) (41).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by The ethics committees of CREEA (Rennes Committee of Ethics in Animal Experimentation) and of the France's Ministry of Higher Education and Research approved the entire protocol (authorization #2016011111546978).

AUTHOR CONTRIBUTIONS

IL, SB, DD, PL, and CB conceived the project and ML, IL, SB, DD, PL, and CB designed the experiment. IC, PL, and CB provided the infant formulas. ML, OM, AC, IN, VB-B, BC, AD, IL, and SB performed analysis. ML, OM, BC, AD, DD, SB, and IL analyzed the data. ML, AD, SB, and IL wrote the draft manuscript. All authors reviewed and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.615248/full#supplementary-material

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Heat-Treatments Affect Protease Activities and Peptide Profiles of Ruminants' Milk

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Proteases present in milk are heat-sensitive, and their activities increase or decrease depending on the intensity of the thermal treatment applied. The thermal effects on the protease activity are well-known for bovine milk but poorly understood for ovine and caprine milk. This study aimed to determine the non-specific and specific protease activities in casein and whey fractions isolated from raw bovine, ovine, and caprine milk collected in early lactation, and to determine the effects of low-temperature, long-time (63°C for 30 min) and high-temperature, short-time (85°C for 5 min) treatments on protease activities within each milk fraction. The non-specific protease activities in raw and heat-treated milk samples were determined using the substrate azocasein. Plasmin (the main protease in milk) and plasminogen-derived activities were determined using the chromogenic substrate S-2251 (D-Val-Leu-Lys-pNA dihydrochloride). Peptides were characterized using high-resolution liquid chromatography coupled with tandem mass spectrometry. The activity of all native proteases, shown as non-specific proteases, was similar between raw bovine and caprine milk samples, but lower ($P < 0.05$) than raw ovine milk in the whey fraction. There was no difference ($P > 0.05$) between the non-specific protease activity of the casein fraction of raw bovine and caprine milk samples; both had higher activity than ovine milk. After 63°C/30 min, the non-specific protease activity decreased (44%; $P > 0.05$) for the bovine casein fraction only. In contrast, the protease activity of the milk heated at 85°C/5 min changed depending on the species and fraction. For instance, the activity decreased by 49% for ovine whey fraction, but it increased by 68% for ovine casein fraction. Plasmin and plasminogen were in general inactivated ($P > 0.05$) when all milk fractions were heated at 85°C/5 min. Most of the peptides present in heat-treated milk were derived from β-casein and α_{S1}-casein, and they matched the hydrolysis profile of cathepsin D and plasmin. Identified peptides in ruminant milk samples had purported immunomodulatory and inhibitory functions. These findings indicate that the non-specific protease activity in whey and casein fractions differed between ruminant milk species, and specific thermal treatments could be used to retain better protease activity for all ruminant milk species.

Keywords: plasmin, plasminogen, cathepsin D, bovine milk, ovine milk, caprine milk, peptides

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INTRODUCTION

Proteases are degradative enzymes found in plants, microorganisms, and animals; they hydrolyze proteins into peptides and amino acids (1). Proteases present in milk (plasmin, elastase, cathepsin D, and carboxy- and aminopeptidases) produce and control the hydrolysis of peptides before and during gastrointestinal digestion, promoting bioactivities (e.g., sleep induction, mucosal development, and immunomodulatory and gastrointestinal functions) and helping with digestion within the gastrointestinal tract (2–4). The latter is important for infant when the gastrointestinal tract is immature, and milk enzymes appear to help the infant to digest milk proteins.

Most of the proteases in bovine milk are secreted in their inactive form (zymogens) and become active by cleavage of a specific peptide bond by an activator (5). A balance between protease activators and inhibitors is essential to retain adequate peptide and amino acid profiles for optimal digestion, absorption, and function in the gastrointestinal tract (4).

Thermal processes used in the food industry can affect protease activities in milk (6). Most of the components (zymogens, active enzymes, activators, and inhibitors) in the proteolytic system are heat-sensitive and can be inactivated during ultra-high-temperature (UHT) conditions (e.g., 143°C for 15 s). However, mild treatments, such as pasteurization (e.g., 72°C for 15 s), do not completely inactivate some zymogens and their activators. Thus, mild treatments can result in higher protease activity in pasteurized milk when compared with their raw or UHT counterparts, due to destabilization of proteolytic systems and conversion of zymogens into an active form without the interference of inhibitors (6–9).

The protease activity and the thermal effects on the protease activity are well-known for bovine milk. For instance, bovine casein fraction has a higher concentration of plasmin and plasminogen than the bovine whey fraction (10). However, the protease activity and the thermal effect on the protease activity in other ruminant milk (ovine and caprine) fractions are little studied. Other ruminant milk species have different protein content, amino acid sequences, casein-to-whey protein ratio, and micelle structure to bovine milk (11). Given these differences, the study hypothesized that thermal treatments applied to milk from different ruminant species might yield different protease activities and peptide sequences.

Thus, this study aimed to determine protease activities in casein and whey fractions from bovine, ovine, and caprine raw milk samples collected in early lactation, and to investigate the effect of thermal treatments (63°C for 30 min and 85°C for 5 min) on protease activities within each fraction. The non-specific protease activities of bovine, ovine, and caprine skim milk samples, either raw or heat-treated, were determined using the substrate azocasein. Plasmin and plasminogen activities were determined using the chromogenic substrate S-2251 (D-Val-Leu-Lys-pNA dihydrochloride). Peptides were characterized by peptidomics using high-resolution liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).

MATERIALS AND METHODS

Milk Sampling

Fresh raw bovine, ovine, and caprine milk samples were collected at early lactation to avoid the variation of enzyme activities due to lactation stage as reported previously (12). Early lactation was defined as the period between 14 and 50, 35, and 40 post-partum days for bovine, ovine, and caprine, respectively (12–14). Raw bovine milk was provided by Dairy 4 Farm, Massey University (Palmerston North, New Zealand), and ovine and caprine milk samples obtained from local dairy farms within 10 km to Palmerston North, New Zealand. Milk heat-treatments were carried out in the morning on the same day as collection. For each species, three batches of milk from different animals were collected and heat-treated over three separate days (i.e., $n = 3$ biological replicates).

Heat-Treatments

Ruminant milk samples were first skimmed at 2455 *g* for 30 min at 4°C to remove the fat. Skim milk samples were then heat-treated at 63°C for 30 min and 85°C for 5 min using a thermostatically controlled water bath with shaking. These thermal conditions were chosen to represent the conventional pasteurization using low-temperature, long-time (LTLT; 63°C for 30 min) and high-temperature, long-time (HTLT; 85°C for 5 min) treatments. The heating was performed in a 150-ml capped sterile bottle, and the temperature was monitored through a thermometer (MS6514, Mastech, Dongguan, China) inserted at the center of the liquid. After holding the milk for the required temperature and time, the milk samples were rapidly cooled down in an ice-water bath until the temperature reached approximately 10°C. Raw and heat-treated samples were aliquoted and frozen at −20°C and analyzed within 1 week. Samples were frozen to avoid protein hydrolysis during cold storage (5°C) as previously reported (15, 16). Raw milk samples were used as controls.

Preparation of Casein and Whey Milk Fractions

The casein and whey fractions were separated by ultracentrifugation to determine the protease activity of each fraction. Raw and heat-treated milk samples were thawed and ultracentrifuged at 100,000 × *g* for 1 h at 20°C. The supernatant (milk whey fraction) and pellet (casein micelles) were separately collected. The casein fraction was then prepared by suspending the casein micelles in pH 8.0 buffer (50 mM Tris-HCl, 110 mM NaCl, and 50 mM ε-amino-n-caproic acid) as previously described (5). The suspended casein fraction was incubated at room temperature for 2 h to dissociate plasmin and plasminogen from the casein micelles. The casein suspension was then ultracentrifuged at 100,000 × *g* for 1 h at 20°C, and the supernatant (or casein fraction) was used to determine the non-specific protease, plasmin, and plasminogen-derived activities in raw and pasteurized (63°C/30 min and 85°C/5 min) ruminant's milk.

Determination of Non-specific Protease Activity

Non-specific protease activity of both casein and whey fractions isolated from raw and heated ruminant milk samples were determined as previously described (17–19) using azocasein (a protein coupled with diazotized aryl amines) as substrate.

Briefly, 1 ml of 1% azocasein solution (catalog A2765, Sigma-Aldrich, St. Louis, USA) was combined with 100 μl of casein or whey fraction. Samples were incubated at 36°C for 15 min, and the reaction was stopped by the addition of 2 ml of 5% TCA solution (catalog T6399, Sigma-Aldrich, St. Louis, USA). Samples were centrifuged at 2455 *g* at room temperature for 4 min, and the supernatant was filtered on paper Whatman 1 (Sigma-Aldrich, St. Louis, USA). The absorbance of the filtrate was read at 345 nm in a UV/Visible spectrophotometer (Genesys, Thermo Fisher Scientific, Massachusetts, USA).

Absorbance values were compared to a standard calibration curve, which was generated by mixing 100 μl of protease from *Bacillus* sp. solution (16 U/g, catalog P3111, Sigma-Aldrich, St. Louis, USA) at different concentrations with 1% azocasein solution. The absorbance was determined as described above at 345 nm, and the coefficient of determination (r^2) obtained was 0.9991. The analysis was performed in triplicate.

Determination of Plasmin and Plasminogen Activities

Plasmin (and its zymogen plasminogen) is the major enzyme found in bovine milk. In this study, plasmin was chosen as a model milk enzyme to compare its activity across ruminant milk samples and thermal processes. The plasmin and plasminogen-derived activities naturally present in casein and whey milk fractions were determined in all samples using the method previously described (20, 21).

Plasminogen was converted into active plasmin during a 60-min incubation at 37°C of 500 μl of milk fraction (casein or whey) in the presence of 500 μl of urokinase solution (catalog U4010, Sigma-Aldrich, St. Louis, USA; 200 Ploug U/ml prepared with 100 mM Tris–HCl, pH 8).

Plasmin and plasminogen-derived activities, after plasminogen activation by urokinase, were determined by measuring the rate of hydrolysis of chromogenic substrate S-2251 (H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide dihydrochloride, catalog V0882, Sigma-Aldrich, St. Louis, USA) and release of *p*-nitroanilide. The method involves the reaction of 100 μl of the sample with 100 μl of buffer (100 mM Tris-HCl, 4 mM S-2251).

The absorbance at 405 nm of the milk samples was measured at 30-min intervals for 120–180 min, depending on the level of plasmin activity in the sample, in a multi-mode microplate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany) at 37°C. The plasminogen-derived activity was calculated as the difference between the plasmin activity before and after plasminogen activation with urokinase. Plasmin activity was expressed in *p*-nitroanilide units (micromoles of *p*-nitroanilide released per minute) per milliliter of milk. The analysis was performed in triplicate.

Peptide Characterization via LC-MS/MS

Peptide Extraction

The raw and heat-treated ruminant milk samples were analyzed by LC-MS/MS to determine the peptide sequences as described elsewhere (22, 23). Milk samples (500 μl) were subjected to acid precipitation with HCl (pH 4.5) at room temperature for 1 h. The milk samples were centrifuged at 14,000 × *g* for 25 min at 4°C. An aliquot of whey was taken from the middle of the supernatant. After removing the remaining supernatant, casein (pellet) was obtained. This fractionation method was different from the method used above, as here it was not required to dissociate the enzymes from casein micelles. This fractionation was conducted to be able to identify peptides with low abundance.

Casein was dissolved in 100 μl of 5% acetonitrile using ultrasonication. Following centrifugation, at 14,000 × *g* for 25 min at 4°C, 100 μl of whey and casein fractions was ultrafiltered individually using 10-kDa NanoSep centrifugal ultrafilters (Pall, Ann Arbor, Michigan, USA). The ultrafiltrates were then dried in a vacuum centrifuge and resuspended in 100 μl of 0.1% formic acid.

Data Acquisition

Mass spectrometry was carried out on a nanoflow Ultimate 3,000 UPLC (Thermo Scientific, San Jose, CA) coupled to Impact II mass spectrometer with a CaptiveSpray source equipped with a nanoBooster device (Bruker Daltonik, Bremen, Germany) operated at 1,800 V. For each fractionated sample, 1 μl of the sample was loaded on a C18 PepMap100 nano-Trap column (300 μm ID × 5 mm, 5 micron 100 Å) at a flow rate of 3,000 nl/min. The trap column was then switched in line with the analytical column ProntoSIL C18AQ (100 μm ID × 150 mm 3 micron 200 Å) (nanoLCMS Solutions, Gold River, CA). The reverse-phase elution gradient was from 2 to 20% to 45% solvent B over 60 min, total 88 min at a flow rate of 600 nl/min. Solvent A was LCMS-grade water with 0.1% formic acid; solvent B was LCMS-grade acetonitrile with 0.1% formic acid.

The fractionated samples were measured in data-dependent MS/MS mode, where the acquisition speed was 2 Hz in MS and 2–32 Hz in MS/MS mode depending on precursor intensity. Ten precursors were selected in the m/z 150–2,200 range, with one to eight charged peptides selected. The analysis was performed in positive ionization mode with a dynamic exclusion of 60 s.

Peptide Identification

The PEAKS × Studio data analysis software package (Bioinformatics Solutions Inc, Waterloo, Canada) was used to analyze the LC-MS/MS data. The raw data were refined by a built-in algorithm that allows the association of chimeric spectra. The peptides were identified with the following parameters: a precursor mass error tolerance of 10 ppm and fragment mass error tolerance of 0.05 Da were allowed; peptides with a length starting at four amino acids long were included; the UniProt *Ovis aries* database (v2019.08, 27,855 sequences), UniProt *Bos taurus* database (v2019.08, 46,707 sequences), and UniProt *Capra hircus* database (v2019.08, 35,307 sequences) were used; and no enzyme was specified as a digestive enzyme. Oxidation (M), phosphorylation (STY), and deamidation

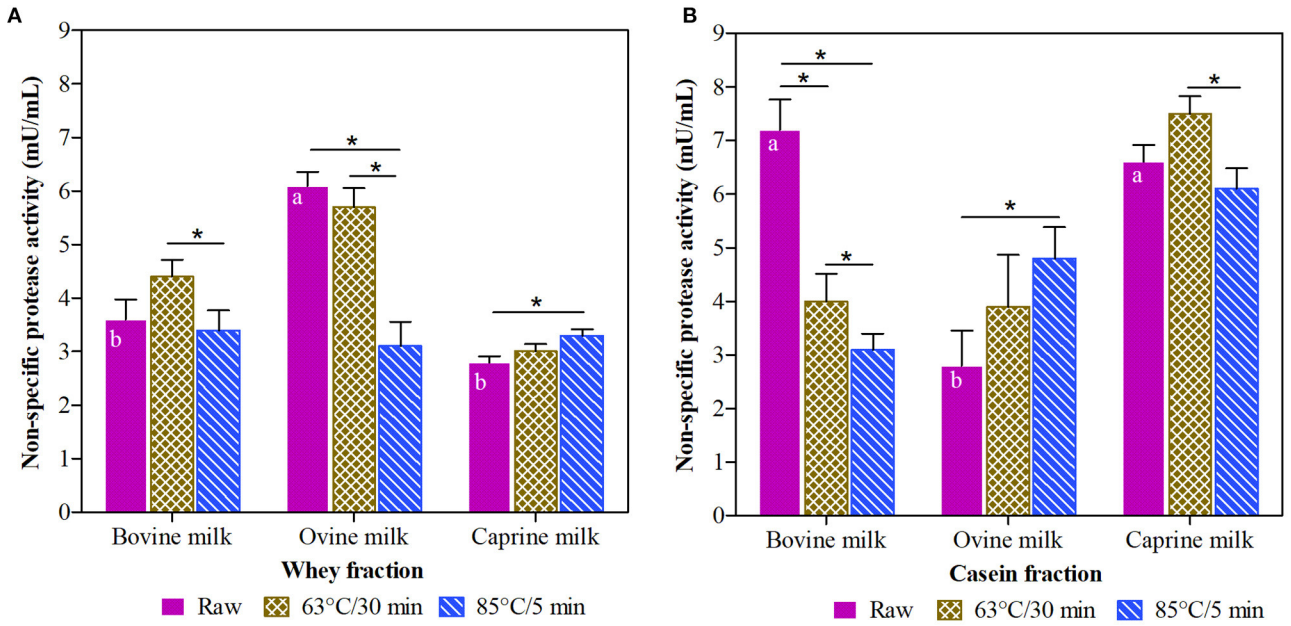

FIGURE 1 | Non-specific protease activity in whey **(A)** and casein **(B)** fraction of ruminant milk samples (bovine, ovine, and caprine) unheated (raw) and heated (63°C/30 min and 85°C/5 min). There was a significant effect ($P < 0.001$) for all main factors (species, treatments, and fractions) and all their interactions. Values are means ± SEs, $n = 3$. Means with different letters across species (bovine, ovine, and caprine) within raw milk for each fraction differ significantly ($P < 0.001$). Lines with an asterisk represent significant differences ($P < 0.05$) between treatments (raw, 63°C/30 min and 85°C/5 min) within each species. For raw bovine milk and raw and heated (63°C/30 min and 85°C/5 min) ovine and caprine milk, there were differences between the whey and casein fraction ($P < 0.001$).

(NQ) were chosen as variable modifications in Peaks DB, and unexpected modifications were accounted for in the Peaks PTM search module. A maximum of three post-translational modifications (PTMs) per peptide was permitted. False discovery rate (FDR) estimation was made based on decoy fusion. An FDR of <1% with a peptide spectrum match hit and a PTM A-score of 100 was considered adequate for confident peptide identification. The peptides identified in each fraction were then compiled for each milk type and heat-treatment combination. When peptides with the same amino acid sequences and retention time (i.e., redundant peptides) were identified multiple times in the same sample, it was considered as only one peptide. The mass spectrometry data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD022702 (http://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD022702) (24).

Comparison of Peptide Profiles Between Treatments for Each Species

The identified non-redundant peptides were used to identify common peptides between raw and 63°C/30 min, and raw and 85°C/5 min treatments within the species to identify resistant peptides to each thermal treatment.

Enzyme Prediction

The EnzymePredictor tool (http://bioware.ucd.ie/~enzpred/Enzpred.php) (25) was used to predict which proteases were involved in peptide cleavage for each milk type and heat-treatment combination. The tool was built to identify the action of 35 enzymes, which are from endogenous, bacterial, and digestive system sources. Here, only the activity of main native enzymes in milk (plasmin, cathepsin D, and elastase) were identified. The non-redundant peptide sequences organized by treatment and species combinations were uploaded on the EnzymePredictor tool. For each enzyme, the activity was predicted based on the number of peptides cleaved (**Figure 4**).

Bioactive Peptides Prediction

The non-redundant peptide sequences in each treatment and species were used to predict the potential bioactive properties using the Milk Bioactive Peptide Database (MBPDB) (http://mbpdb.nws.oregonstate.edu/) (26). The MBPDB compiles a comprehensive database of functional peptides in milk from mammalian species across the available literature sources, allowing comparison of known functional peptides with biological datasets to explore the presence of potential bioactive peptides in food sources.

In this study, the query batch of peptide sequences for each treatment and species was set to find the exact match in the MBPDB database without distinction of species. A list of bioactive peptides was obtained for each sample to identify potential bioactive peptides and compare them between treatments within the same species.

Statistical Analysis

Statistical analyses were performed using the Mixed Model procedure of SAS (SAS/STAT version 9.4; SAS Institute Inc., Cary, NC, USA). A 3 × 2 × 2 factorial model was used to test the effect of milk treatments (raw, 63°C/30 min and 85°C/5 min), fractions (casein and whey), species (bovine, ovine and caprine) on non-specific protease, plasmin, and plasminogen activities.

The model diagnostics for each response variable were tested after combining the Output Delivery System Graphics procedure and the Repeated statement of SAS, before comparing the means. The repeated statement in the Mixed Model procedure was used to test the homogeneity of variances by fitting models with the Restricted Maximum Likelihood method and comparing them using the log-likelihood ratio test. Each response variable in the selected model had adjusted equal variances across treatments. Selected means were compared using adjusted Tukey test when the F value of the analysis of variance was significant ($P < 0.05$).

RESULTS

Heat-Treatments Affect Non-specific Protease Activity in Ruminant Milk Fractions

This study reports the effect of different thermal treatments (raw, 63°C/30 min and 85°C/5 min) on non-specific protease activity of casein and whey fractions isolated from ruminant milk (bovine, ovine, and caprine) samples (**Figure 1**). There was a significant interaction between species, heat-treatments, and fractions ($P < 0.001$).

Raw milk from bovine and caprine species had higher non-specific protease activity in the casein than whey fraction. For example, raw caprine milk showed 48% higher non-specific protease activity in casein than whey fraction. However, in the whey fraction, the non-specific protease activity of raw ovine milk samples showed, on average, 25% higher ($P < 0.05$) activity than raw bovine milk and raw caprine milk samples (**Figure 1A**).

The intensity of the thermal treatments applied to ruminant milk samples affected the non-specific protease activity differently in whey and casein fractions. Compared with their raw fractions, the non-specific protease activity in the bovine casein fraction was reduced ($P < 0.05$) by 44 and 71% after the thermal treatments at 63°C/30 min and 85°C/5 min, respectively (**Figure 1B**), whereas, these thermal treatments did not affect the non-specific protease activity of the bovine whey fraction (**Figure 1A**).

For the whey fraction of ovine milk, the thermal treatment at 63°C/30 min was more effective in retaining the non-specific protease activity compared to 85°C/5 min. For raw caprine milk, neither of the thermal treatments significantly affected ($P > 0.05$) the non-specific protease activity in the casein fraction (**Figure 1B**). From the raw fractions, the thermal treatment at 85°C/5 min significantly increased ($P < 0.05$) the non-specific protease activities in casein fraction of ovine (68%) (**Figure 1B**) and whey fraction of caprine (12%) milk (**Figure 1A**).

Heat-Treatments Affect Plasmin and Plasminogen-Derived Activity in Ruminant Milk Fractions

Raw ovine whey fraction had 4-fold and 11-fold higher plasmin activity (**Figure 2A**) than their matched raw caprine and bovine whey fractions, respectively.

The thermal treatment at 85°C/5 min of the raw milk resulted in a significant decline (93 to 100%) of plasmin activity in both whey and casein milk fractions of all species (**Figures 2A,B**). In contrast, the thermal treatment at 63°C/30 min resulted in significantly increased plasmin activity in ovine whey and bovine casein fractions (45 and 100% increase, respectively).

Among raw milk samples, the bovine milk had the highest ($P < 0.05$) plasminogen-derived activity in whey and casein fractions, with a 9-fold higher concentration in the casein fraction (**Figures 3A,B**). In general, the milk fractions heated at 63°C/30 min had a similar plasminogen-derived activity compared to their raw counterparts, with the exception of caprine whey and bovine casein fractions. In contrast and in general, the treatment at 85°C/5 min reduced the plasminogen-derived activity up to 100%.

Differences in Endogenous Peptide Profiles and Their Predicted Bioactivity Between Raw and Heat-Treated Ruminant Milk Samples

The peptide sequences that were identified in both raw and 63°C/30 min, and raw and 85°C/5 min, milk samples for each species are shown in **Supplementary Tables 1, 2**. For raw bovine milk, 72 non-redundant peptide sequences were identified, but only three and five of them were also detected after 63°C/30 min and 85°C/5 min, respectively. Other peptides were observed for 63°C/30 min (129) and 85°C/5 min (125).

For ovine milk, there were 95 and 41 common peptide sequences between raw and milk heated at 63°C/30 min and 85°C/5 min, respectively. However, in contrast with bovine milk, the number of non-common peptide sequences between the treatments decreased 13–61% at 63°C/30 min and 39–83% at 85°C/5 min in caprine and ovine milk samples, respectively.

The non-redundant peptide sequences identified in each treatment within the species were analyzed with the EnzymePredictor to predict which milk proteases (plasmin, cathepsin D or elastase) were involved in peptide bond cleavage during the thermal treatments. Similarly, the Milk Bioactive Peptide Database (MBPDB) was used to predict whether the identified peptide sequences in each milk sample matched any peptides reported with bioactivity.

Based on the EnzymePredictor, plasmin was the main protease involved in the protein hydrolysis of heat-treated bovine and caprine milk samples (**Figure 4**). However, for ovine milk, the enzyme cathepsin D (and elastase for 85°C/5 min) was most likely involved in the protein hydrolysis. In general, the intensity of the heat-treatment affected the hydrolysis of proteins, and the use of high-temperature, short-time (85°C/5 min) treatment showed a lower number of peptides cleaved by the three analyzed endogenous enzymes (plasmin, cathepsin D,

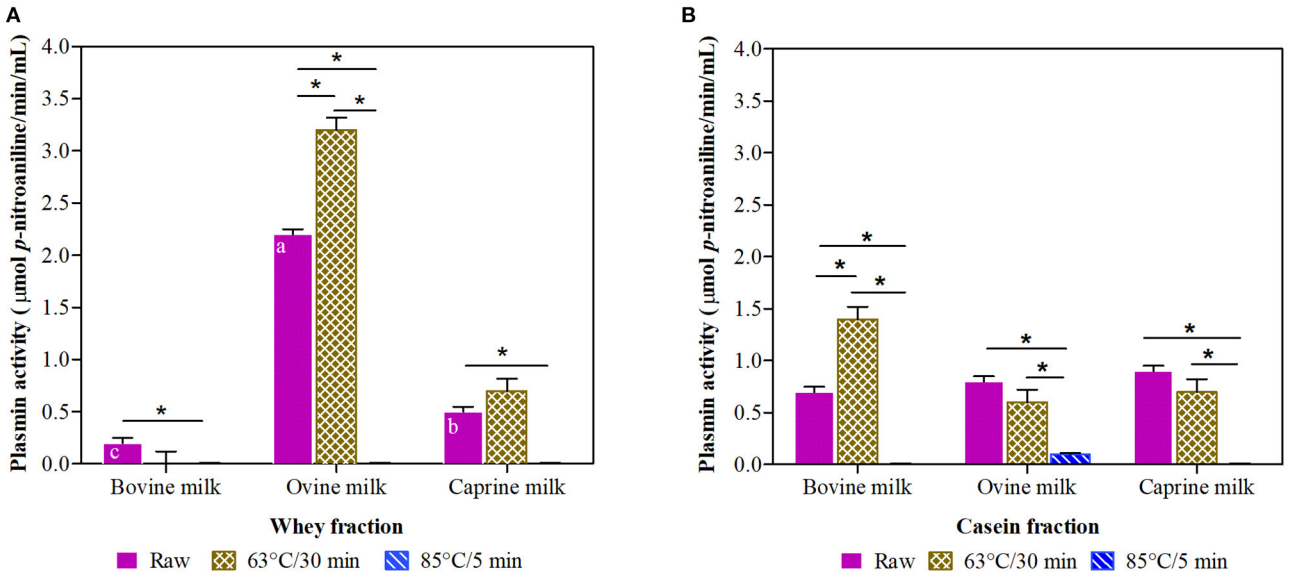

FIGURE 2 | Plasmin activity in whey **(A)** and casein **(B)** fraction of ruminant milk (bovine, ovine, and caprine) unheated (raw) and heated (63°C/30 min and 85°C/5 min). There was a significant effect ($P < 0.001$) for all main factors (species, treatments, and fractions) and all their interactions. Values are means ± SEs, $n = 3$. Means with different letters across species (bovine, ovine, and caprine) within raw milk for each fraction differ significantly ($P < 0.001$). Lines with an asterisk represent significant differences ($P < 0.05$) between treatments (raw, 63°C/30 min and 85°C/5 min) within each species. For raw caprine and raw and heated at 63°C/30 min bovine and ovine milk, there were differences between the whey and casein fraction ($P < 0.001$).

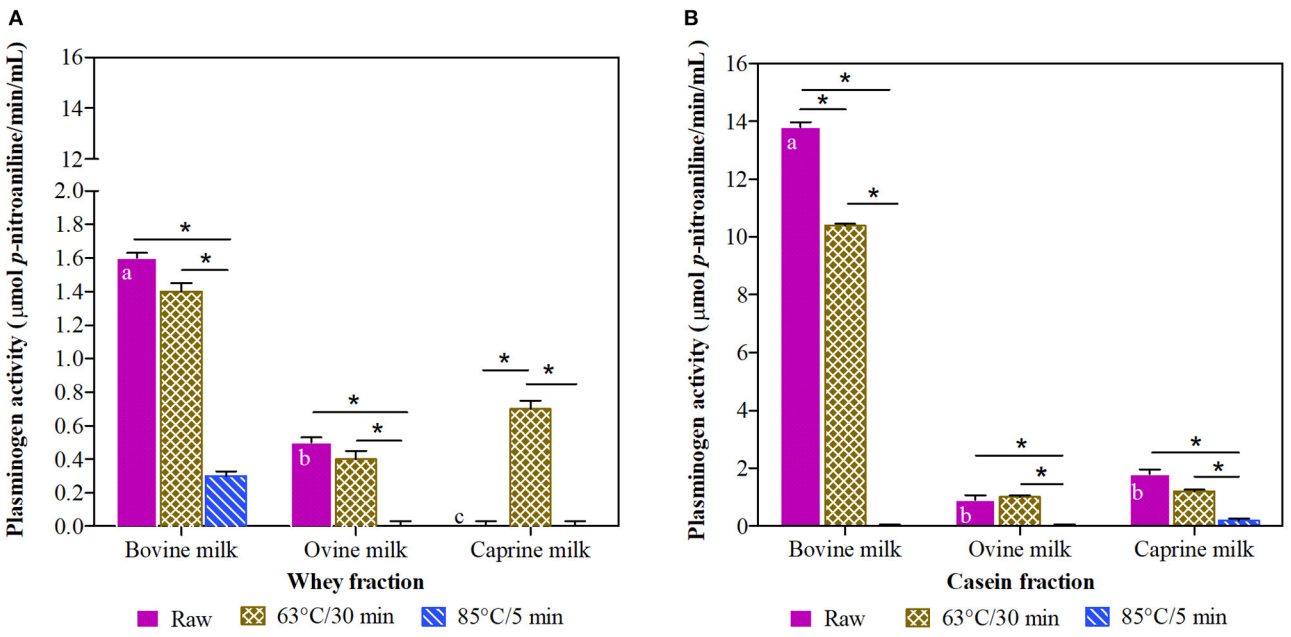

FIGURE 3 | Plasminogen activity in whey **(A)** and casein **(B)** fraction of ruminant milk (bovine, ovine, and caprine) unheated (raw) and heated (63°C/30 min and 85°C/5 min). There was a significant effect ($P < 0.001$) for all main factors (species, treatments, and fractions) and all their interactions. Values are means ± SEs, $n = 3$. Means with different letters across species (bovine, ovine, and caprine) within raw milk for each fraction differ significantly ($P < 0.001$). Lines with an asterisk represent significant differences ($P < 0.05$) between treatments (raw, 63°C/30 min and 85°C/5 min) within each species. For raw and heated (63°C/30 min and 85°C/5 min) bovine milk, raw and heated at 63°C/30 min caprine milk, and heated at 63°C/30 min ovine milk, there were differences between the whey and casein fraction ($P < 0.001$).

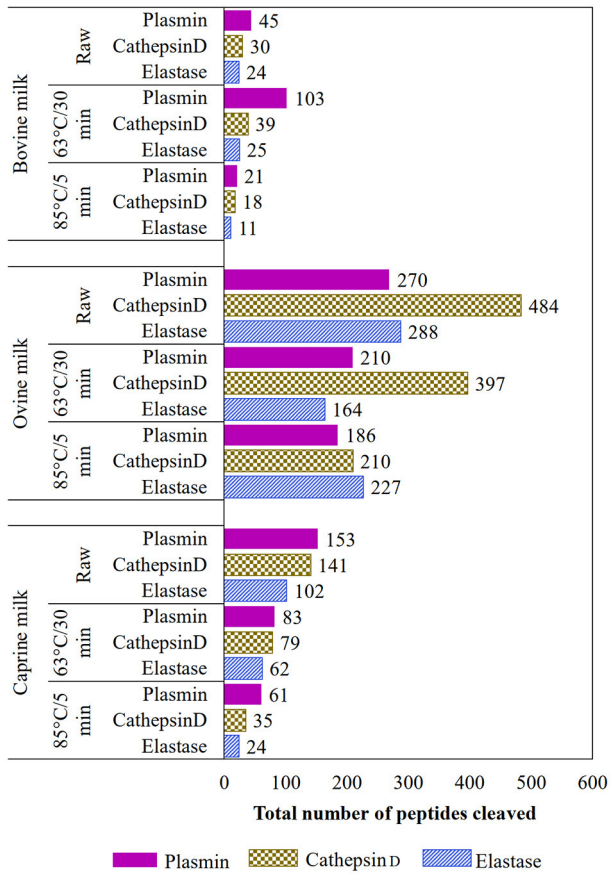

FIGURE 4 | Total predicted number of non-redundant peptides attributed to plasmin, cathepsin D, or elastase in raw and heated (63°C/30 min and 85°C/5 min) ruminants' milk (bovine, ovine, and caprine) using the EnzymePredictor tool.

and elastase) compared with raw and lower-temperature, long-time (63°C/30 min) treatment, at least for bovine and caprine milk types.

The MBPDB revealed that the heat-treatments affected the presence of potential bioactive peptides in ruminant milk samples. For example, raw bovine milk showed a low number (3) of potential bioactive peptides compared with heat-treated milk samples at 63°C/30 min (5) (**Supplementary Table 3**). The potential bioactive peptides identified in bovine milk samples have several purported functions including immunomodulatory, antimicrobial, inhibitory, and cytotoxic. In contrast, for ovine and caprine milk samples, there was a reduction (42–67%) of potential bioactive peptides when the raw milk samples were heat-treated at 63°C/30 min (**Supplementary Table 3**).

DISCUSSION

Studies investigating the distribution of protease activities between milk fractions have been mainly focused on bovine milk, and there is little information reported for caprine and ovine milk. This study is the first to show the effect of different thermal treatments on endogenous protease activities of milk fractions from different ruminant species. In this study, milk casein and whey fractions isolated from bovine, ovine, and caprine milk samples were used to determine differences related to species, and the effect of different thermal treatments (63°C/30 min and 85°C/5 min) on non-specific and specific protease (plasmin) activities. Peptide sequences in raw and heat-treated ruminant milk samples were also determined by high-resolution LC-MS/MS to predict both the native enzymes (plasmin, elastase, and cathepsin D) involved in peptide formation (protein hydrolysis) and potential bioactivity of the identified peptides.

The activity of all native proteases, shown as non-specific proteases, was similar between raw bovine and caprine milk samples, but lower than raw ovine milk samples in the whey fraction. The higher non-specific protease activity observed in raw ovine whey fraction could be associated with the action of cathepsin D, as predicted by the EnzymePredictor, which is the second proteinase with higher activity in milk, and it is normally present in acid whey (27).

The high plasmin activity in raw whey ovine milk samples compared to that in whey bovine and caprine milk samples could be explained by its lower casein:whey ratio (4.7, 3.1, and 3.5 in bovine, ovine, and caprine) and higher protein content (1.6-fold and 1.4-fold higher than bovine and caprine milk) (28).

Thermal treatments are commonly applied to inactivate potential microbial pathogens in manufacturing dairy products and extend their shelf-life. However, as reported previously for bovine milk (29, 30) and observed here for the casein and whey fractions of bovine, caprine, and ovine milk samples, heat-treatments affect the activity of the native enzymes. For instance, and as observed here, higher-temperature, short-time heat-treatment (85°C/5 min) reduced to 93–100% the original plasmin activity in both the casein and whey fraction of all ruminants' milk. According to other studies (31, 32), the components of the plasmin system have variable heat sensitivities. During mild heat-treatment (75°C for 15 s), plasminogen inhibitor loses 81% of its original activity, while plasminogen activators are more heat-stable (*D* value of 32 s at 140°C), increasing the conversion of plasminogen to plasmin. Thus, the activation of plasminogen into plasmin can result from the heat sensitivity of the components, which are part of the complex enzyme system. For example, bovine milk has more plasminogen (zymogen) than plasmin activity in the casein fraction; however, when the raw milk was heat-treated at 63°C/30 min, the plasminogen-derived activity decreased (25%), and the plasmin activity increased (100%), suggesting the conversion of the zymogen to active enzyme during this thermal treatment. This appears to explain both the higher number of predicted peptides cleaved by plasmin in the heat-treated bovine milk (103 cleavages) compared to its raw counterpart (45 cleavages) (**Figure 4**) and the low number of overlapping peptides between heat-treated and raw bovine milk. For instance, the peptide SSRQPQSQNPKLPLSILKEKHL (22 amino acids) identified in raw bovine milk was also identified as 18 (SSRQPQSQNPKLPLSILK) and 10 (LPLSLKEKHL) amino acids at 63°C/30 min.

Similar to the casein fraction of the bovine milk, the plasmin activity of the whey fraction in the ovine milk also increased (45%) after the thermal treatment at 63°C/30 min, but the plasminogen activity was not modified. The reasons for this increase in plasmin activity are unclear.

Mass spectrometry experiments were designed to characterize the endogenous peptides qualitatively. The results were also used to predict the number of peptides cleaved by the main milk proteases. This approach has been used in other studies (25, 33). Care is needed when interpreting the number of cleaved peptides, as it does not account for the total number of peptides in the sample.

The number of non-redundant endogenous peptides identified in this study (72 peptides) using a qualitative peptidomic approach for raw bovine milk is within the range of peptides reported by other researchers: 33 (34) and 159 (35) peptides. However, raw ovine (790 peptides) and caprine (374 peptides) milk showed higher number of peptides than previously reported: 718 (36) and 261 (37) peptides, respectively. The variation in the number of peptides between the studies could be associated with different factors such as the mass spectrometry instrument used, the milk origin, and lactation stage of the milk samples used.

Most of the peptides generated during the heat-treatment of ovine milk at 63°C/30 min were derived from β-casein and were predicted to be mainly hydrolyzed by cathepsin D. This result suggests that although ovine milk had the highest plasmin activity, plasmin was not responsible for most of the hydrolysis that happened during the heat-treatment.

According to the EnzymePredictor tool, the heat-treatment (85°C/5 min) reduced the number of peptide bonds cleaved by plasmin, cathepsin D, and elastase. This reduction can be ascribed to the inactivation of the enzymes during this treatment, which was confirmed when plasmin (active or inactive form) activity was evaluated as a model enzyme. Although the later supports the predicted number of cleaved peptides, further work using quantitative peptides identification is warranted.

Like ovine milk, most of the peptides with potential bioactivity identified in bovine and caprine milk were treated at 63°C/30 min derived from β-casein. However, the predicted hydrolysis was mainly caused by plasmin. Previous studies have reported that most of the peptides in milk are mainly derived from β-casein (35, 38), and this can be ascribed to the structure (primary to quaternary) of this protein, which renders it more susceptible to proteolysis. The hydrolysis of proteins during the thermal treatment releases peptides with different functions than those present in raw milk. For example, ovine milk samples heated at 63°C/30 min have peptides with specific biological activities, such as cytomodulatory and opioid, that were not detected in raw ovine milk samples.

Some of the potential bioactive peptides identified in pasteurized milk, such as with opioid bioactivity, are resistant to gastric conditions (3) and, combined with bioactive peptides released during gastrointestinal digestion, can increase the availability of functional peptides reaching the intestine, where they are then distributed throughout the body by the circulation blood (2). However, future research is needed to determine the bioavailability of the potential bioactive peptides identified in this study.

In conclusion, non-specific and specific protease activities in casein and whey fractions of ruminant milk samples were reduced when high-temperature, long-time (85°C for 5 min) treatment was applied, and this was reflected in the predicted number of peptides broken by the main milk proteases. The use of mild heat-treatments, like 63°C/30 min, preserved the activities of the studied native proteolytic enzymes in ruminants' milk and generated new potential bioactive peptides with different purported functions than those detected in raw ruminant milk samples.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: http://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD022702.

AUTHOR CONTRIBUTIONS

NR and WM developed the research project and sourced the funding. JL, CM, SL, JM, NR, and WM designed the study. JL performed the experiments. JL supported by SL, CM, and JM analyzed the data. JL supported by CM, JM, NR, EM, SL, and WM wrote and edited the paper. CM did the statistical analysis. EM identified the peptides. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.626475/full#supplementary-material

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REVIEW
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The Relationship Between Breast Milk Components and the Infant Gut Microbiota

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The assembly of the newborn's gut microbiota during the first months of life is an orchestrated process resulting in specialized microbial ecosystems in the different gut compartments. This process is highly dependent upon environmental factors, and many evidences suggest that early bacterial gut colonization has long-term consequences on host digestive and immune homeostasis but also metabolism and behavior. The early life period is therefore a "window of opportunity" to program health through microbiota modulation. However, the implementation of this promising strategy requires an in-depth understanding of the mechanisms governing gut microbiota assembly. Breastfeeding has been associated with a healthy microbiota in infants. Human milk is a complex food matrix, with numerous components that potentially influence the infant microbiota composition, either by enhancing specific bacteria growth or by limiting the growth of others. The objective of this review is to describe human milk composition and to discuss the established or purported roles of human milk components upon gut microbiota establishment. Finally, the impact of maternal diet on human milk composition is reviewed to assess how maternal diet could be a simple and efficient approach to shape the infant gut microbiota.

Keywords: milk oligosaccharides, milk bacteria, milk lipids, gut microbiota, maternal diet

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INTRODUCTION

Under normal circumstances, the gut microbiota has a symbiotic relationship with the host. However, many chronic human diseases, including obesity, diabetes, cirrhosis, rheumatoid arthritis, and inflammatory bowel disease, have been associated with alterations in gut microbial communities (1). The infant gut microbiota is shaped in the first thousand days of life (2). Growing body of evidence revealed that altered neonatal colonization and disturbed interactions between the gut microbes and the host during the neonatal period could affect health later in life (3). The microbial colonization process is an orchestrated phenomenon resulting in specialized microbial ecosystems in the different gut compartments. However, this colonization process can be influenced by numerous environmental factors (4). One of the preponderant factors is neonatal diet, and it is largely accepted that human milk (HM) is the optimal diet that stimulates the most adequate microbiota development for the infant. Contrary to what is recommended by the World Health Organization (5), HM is provided for <6 months for a large percentage of children in Western

countries (6). However, even if breastfeeding does not cover the whole microbiota maturation period, breastfeeding status was more associated with the infant gut microbiota composition than solid food introduction in a cohort of 323 healthy infants (7). The importance of HM upon shaping of the infant gut microbiota is also highlighted by the fact that cessation of breastfeeding, rather than introduction of solid food, was required for maturation into an adult-like microbiota in a cohort of 100 Swedish infants (8).

HM is a complex biofluid that provides all the nutrients required to promote infant growth. Beyond HM nutritional properties, the beneficial properties of breastfeeding on risk reduction of infant disease are well-recognized. HM is composed of a large diversity of components classified by their size into two main groups: macronutrients (fat, proteins, and carbohydrates) and micronutrients (vitamins, minerals, etc.), both dispersed between aqueous and colloidal phases (9, 10). HM also contains many bacterial species (11), immunomodulatory components (12), and hormones (13). HM composition is influenced by many factors such as the lactation period, with a different composition whether colostrum (first 48–72 h), transitional milk, and mature milk (from the second week of lactation until the end of lactation) are considered (14). Length of gestation, time of the day, phase of the nursing process (foremilk and hindmilk), and geographical and/or genetic female background also influence HM composition (15–18). Maternal diet also impacts HM composition, mainly fat composition as well as immunomodulatory components and bacterial species, whereas carbohydrate and protein contents seem less sensitive to the maternal diet (19, 20). The beneficial role of HM on gut microbiota development has been mainly attributed to the presence of oligosaccharides (21). However, the contribution of other HM components is also supported by the literature data. Although most of these data are associations between HM components and the infant gut microbiota or are derived from *in vitro* studies, thus not showing causal relationships, they are sometimes supported by human and animal model data. Moreover, most of the HM compounds, except milk oligosaccharides, are likely to be digested and absorbed before reaching the colon. However, a small fraction of the nutrients escapes small intestinal digestion. The amount of total lipids and proteins that reaches the colon under physiological conditions in adults has been evaluated to be between 5 and 8 g per day for dietary lipids (22) and 2–5 g per day for dietary proteins (23). In infants, data are scarce, but piglet studies revealed the presence of small fractions of dietary di- and monoacylglycerides and polar lipids as well as dairy proteins, either intact or as peptides in the ileum of piglets (24). Thus, a role of these HM compounds' fraction on infant colonic microbiota can be purported. The objective of this review is therefore to present the available data suggesting a role of various HM components on shaping the infant gut microbiota. The second objective is to evaluate how maternal diet, through its effect on these HM components, could be a potential leverage to orientate the infant gut microbiota and ensure optimal health.

HUMAN MILK COMPOSITION

Macronutrients

HM macronutrients are composed of lipids, proteins, and carbohydrates. Their concentrations vary over the lactation period from colostrum to mature milk (**Figure 1**): lipids and lactose content increase while proteins and oligosaccharide content decrease mainly during the first month of lactation and very slightly during mature milk stage (25, 26). Macronutrient concentration and type, especially lipid and protein contents, are slightly variable due to the multiple factors impacting HM composition including lactation time, feeding time, or mother's diet for example (15).

Lipids

Lipids are the main macronutrient in terms of energy. They represent 40–60% of energy in mature milk (26–28) and are the second most abundant macronutrient. They provide essential nutrients like polyunsaturated fatty acids and complex lipids (29). Lipids consist of 98% of triacylglycerides; the remaining is composed of diacylglycerides, monoacylglycerides, free fatty acids, phospholipids, and cholesterol. HM contains more than 200 fatty acids present in different concentrations (18). Oleic, palmitic, and linoleic acids, respectively, located in sn-1, sn-2, and sn-3 positions of triacylglycerides (30), are the highest concentrated ones. HM lipids are endogenously produced by the mammary gland or derived from maternal plasma (31). HM fat is packaged into lipid globules with triacylglycerols found in the core and surrounded by a bulk of phospholipids (32). The diameter of milk fat globules varies from 1 to 10 μm with an average diameter of 4 μm in mature milk (33).

Lipid content and composition are affected by many parameters: (i) feed phase (foremilk or hindmilk), respectively, 32 and 56 g/L (34, 35); (ii) lactation stage, lipid content being greater in mature milk than in colostrum; and (iii) maternal diet, which does not impact lipid content but impacts fatty acid profile and particularly that of the long-chain polyunsaturated fatty acids (17, 19, 31).

Proteins and Nitrogen

HM contains a wide range of proteins classified into three major classes: whey proteins, caseins, and mucins. Whey protein is the major fraction of HM proteins and is mostly represented by α-lactalbumin, lactoferrin (LF), lysozyme, and secretory immunoglobulin A (SIgA) (see below for their specific immunity role). α-Lactalbumin is involved in lactose synthesis (36) and has an amino acid composition similar to the amino acid requirement of the infant (37). Casein fraction includes α-, β-, and κ-casein with a predominance of β- and κ-casein (12). They are the main sources of minerals for the infant, including calcium and phosphorus. Casein function is mainly nutritive (38). The whey protein:casein ratio varies with lactation stage from 90:10 in colostrum to 60:40 in mature milk (12, 37, 39). Moreover, total protein level decreases from the first to sixth month of lactation (18). Protein content contributes to the infant growth, particularly by providing essential amino acids, and participates

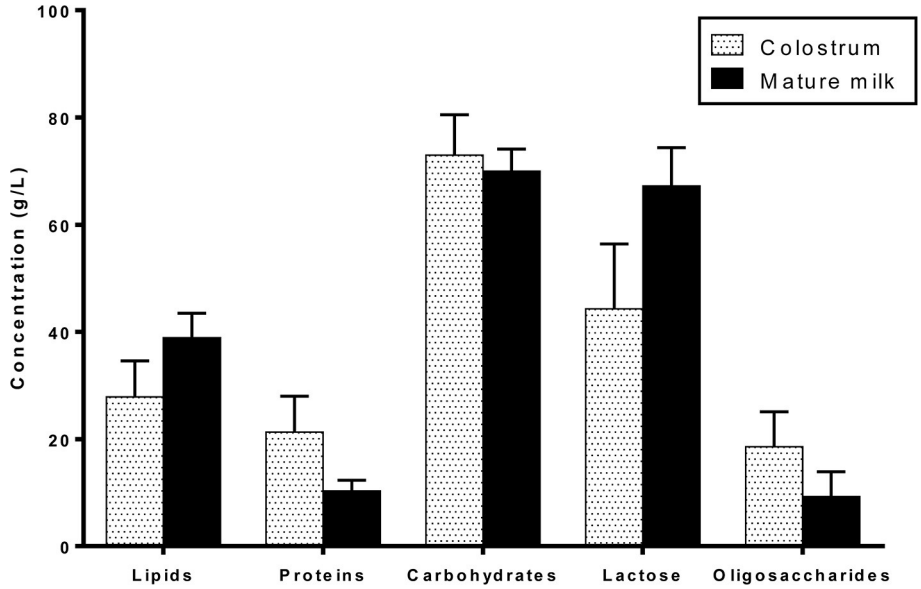

FIGURE 1 | Macronutrient composition of colostrum and mature term human milk.

in immune protection and gut development (25, 39). HM also contains mucins, which belong to the glycoprotein family and are located in the milk fat globule membrane (25). Mucins 1 and 4 are the most studied mucins. Finally, 600 peptides have been recently identified in HM, which have an array of bioactive functions, including antimicrobial activity (40, 41).

Proteins and Peptides With Immunomodulatory and Growth Promotion Activities

LF (20% of total proteins) is found at high concentrations (5 g/L) in colostrum compared with mature milk (3 g/L). LF is a multifunctional protein of the transferrin family and is widely represented in various secretory fluids, like HM (42). LF has both bacteriostatic and bactericidal activities, limiting the growth of several pathogens and killing others. SIgAs in HM are one of the most abundant Igs (43) and the predominant antibody-mediated immune protection in mucosal surfaces of suckling infants. SIgA concentration is high in colostrum (5 g/L) and decreases in mature milk (1.5 g/L) (44). SIgAs provide specific protection against pathogens to which the mother has been previously exposed, via the entero-mammary pathway (45, 46). Activated B cells differentiate into plasma cells that synthesize high-affinity dimeric IgA in the mammary gland, transported into HM across epithelial cells by the polymeric Ig receptor (pIgR) (47). SIgAs may also inactivate viruses (e.g., rotavirus and influenza) within epithelial cells and carry these pathogens and their products back into the lumen, thereby avoiding cytolytic damage to the epithelium. Lysozyme (0.32 g/L in colostrum), another major component in HM, is an enzyme capable of degrading the outer cell wall of Gram-positive bacteria (48).

Cytokines, present in picograms in HM, are small soluble glycoproteins that act as autocrine–paracrine factors by binding to specific cellular receptors, operating in networks and orchestrating immune system development and function (49). They act as messengers to boost the neonatal immune system by communicating with other immune components (50). More particularly in colostrum but also in mature HM, a range of inflammatory cytokines are present in free forms, such as interleukin (IL)-1β, IL-6, IL-8, IL-12, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ, and potentially enhance inflammation (i.e., following bacterial lipopolysaccharides) unlike the immunosuppressive cytokine IL-10, which decreases such inflammatory conditions (51). The primary source of these cytokines is the mammary gland, but leukocytes recovered from HM are capable of secreting them (52). HM also contains an ensemble of growth factors, present at very high concentrations after birth but whose concentrations generally decrease during lactation. Some of these growth factors favor the proliferation and differentiation of epithelial cells and modulate mucosal immune response, such as transforming growth factor (TGF)-β (1–2 μg/L), which is one of the most abundant in HM (53). TGF-β is also an immunosuppressive cytokine involved in the induction and function of regulatory T cells, as well as the regulation of other immune cells such as lymphocytes, macrophages, and dendritic cells, which could induce excessive inflammatory responses to stimuli in the infant gut (53). Colostrum TGF-β is involved in switching IgM to IgA in B lymphocytes of the infant gut mucosa (54).

Non-protein Nitrogen

The nitrogen HM content is also composed of non-protein nitrogen (NPN), which represents 5–10% and 20–25% of the total nitrogen in colostrum and mature HM, respectively. It is composed of urea, creatinine, nucleotides, choline and amino alcohols, amino sugars, carnitine, polyamines (see *Metabolites*

and Bacterial Metabolites section), N-glycans (see *Carbohydrates* section), free amino acids, and peptides (55). Large individual differences in NPN content in HM are observed, likely because this fraction is composed of a heterogeneous mixture of N-containing substances, such as free amino acids that are known to be influenced by several maternal variables. The origin of many NPN compounds in HM is thought to be the metabolic breakdown products, which filter directly from the maternal plasma and/or derive from normal or pathological metabolism within the mammary gland itself (56). Nucleotides are likely to originate from intact or lysed cells in HM. The exact role of most of the NPN compounds is not yet fully established.

Carbohydrates

Lactose

Lactose is the major constituent and the main carbohydrate of HM. It represents 30–40% of HM energy content (57). Lactose concentration increases with lactation stage, with the lowest concentration (around 56 g/L) in colostrum to reach an average content of 69 g/L at 120 days postpartum (58). Nevertheless, lactose has the least variable concentration among HM macronutrients throughout lactation.

Oligosaccharides

HM oligosaccharides (HMOs) are the third major constituent of HM. The amount of HMOs is generally higher in the early stages of lactation, from 20 to 25 g/L in colostrum to 5–15 g/L in mature milk (59–61).

HMOs are defined as unconjugated molecules with a high level of structural diversity as well as major properties and functions (62, 63). All HMOs contain the disaccharide lactose, branched at the reducing and/or non-reducing ends by a single residue or more, generating more than 100 structurally distinct oligosaccharides. The reducing end glucose (Glc) can be fucosylated in α1-3 linkage, while the non-reducing end galactose (Gal) can be fucosylated in α1-2 linkage, sialylated in α2-3 or α2-6, or even elongated in β1-3 by lacto-*N*-biose I (Galβ1,3-GlcNAc) or in β1-6 by *N*-acetyl-lactosamine (Galβ1,4GlcNAc). Additional branching can occur with fucose, sialic acid (Neu5Ac), and/or *N*-acetyl-lactosamine. Thus, HMOs are named as fucosylated neutral HMOs, non-fucosylated neutral HMOs, and sialylated HMOs. Fucosylated and non-fucosylated neutral HMOs encounter 35–50% and 42–55% of total HMOs, respectively (64). Despite the identification of so far more than 150 structurally different HMOs, the main fraction (∼90%) is composed of >20 different ones (65–67) (**Figure 2**).

Overall, the composition in HMOs in HM depends on genetic and environmental factors. The most important variability in HMO composition remains the genetic capacity of individual women to express α1-2-fucosyltransferase FUT2 (secretor gene, *Se*) and/or α1-3/4-fucosyltransferase FUT3 (Lewis gene, *Le*) in the mammary gland (2, 64). Fucosyltransferase (FUT-2 and/or FUT-3) polymorphisms result in four distinct milk groups: Se+Le+, Se–Le+, Se+Le–, and Se–Le–, which, respectively, represented 72–75, 11–18, 7–11, and 3.5% of European or Brazilian mothers (66, 67). The composition in HMOs in the milk of Se+Le+ mothers presents a higher diversity than the composition of HMOs in the milk of Se–Le– ones. Moreover, non-secretor mothers (Se–Le+ and Se–Le–) secrete a lower amount of HMOs than secretor ones (66, 67). The quantification of 20 HMOs from the milk of 290 European mothers during the first 4 months of lactation showed that 2′-fucosyllactose (FL) and lacto-*N*-fucopentaose (LNFP) I are the most abundant oligosaccharides in milk from secretor mothers (67). On the other hand, the highest oligosaccharides in milk of non-secretor mothers are, respectively, 3′-FL/LNFP II and lacto-*N*-tetraose (LNT)/disialyllacto-*N*-tetraose (DSLNT) (67). However, the genetic mother status (*Se/Le*) does not affect the concentrations of 3′-sialyllactose (SL) and lacto-*N*-neodifucohexaose (LNnDFH) as well as the total neutral core and the total acidic HMOs (66, 67). Despite these general profiles, HMO concentrations present a great variability even in the milk of mothers with the same *Se/Le* status (66). Beside genetic factors, time and mode of delivery also affect the amount and the composition of HMOs. The milk of women with preterm infants is overrepresented by sialylated HMOs, and the concentrations of total HMOs are lower than those of women with term infants (64, 68). Samuel et al. showed that the composition of HMOs is also affected by the mode of delivery at day 2 and day 30 of lactation, specifically with a lower amount of 2′-FL, 3′-SL, and 6′-SL in the milk of women who gave birth through caesarian section (67).

In addition to free oligosaccharides, N-glycans are oligosaccharides attached to the asparagine residues of a protein via *N*-acetylglucosamine linkages. Glycosylation is an important post-translational modification of proteins. More than 70% of HM proteins are highly glycosylated (69). They include LF, lactadherin, SIgAs, mucins, α-lactalbumin, various Igs, and at least 26 other proteins in the whey fraction (70).

Micronutrients

HM micronutrients include vitamins and minerals. Vitamins provided by HM are all the essential vitamins needed for infant growth. Vitamin composition is linked to maternal nutritional status, specifically liposoluble vitamins like vitamins A and D (10). Furthermore, breastfeeding provides a wide range of trace elements (copper, zinc, barium, iron, cobalt, manganese, cesium, etc.) to the infant. Their concentrations vary throughout lactation and are higher in colostrum than in mature milk, but their concentration is not affected by maternal intake (9, 10).

Hormones

Many hormones are present in HM, the vast majority being transported into HM from the maternal circulation but several of them being also synthetized within the mammary gland (13, 71). In general, their concentrations in HM are higher than in plasma and in colostrum and transition milk than in mature milk. Their structure may differ from that in plasma due to glycosylation or phosphorylation within the mammary gland before secretion into HM (13). HM hormones include pituitary (prolactin, growth hormone, and thyroid-stimulating hormone), hypothalamus (thyroid-releasing hormone, luteinizing hormone-releasing hormone, somatostatin, gonadotropin-releasing hormone, and growth hormone-releasing hormone), thyroid (thyroxine and

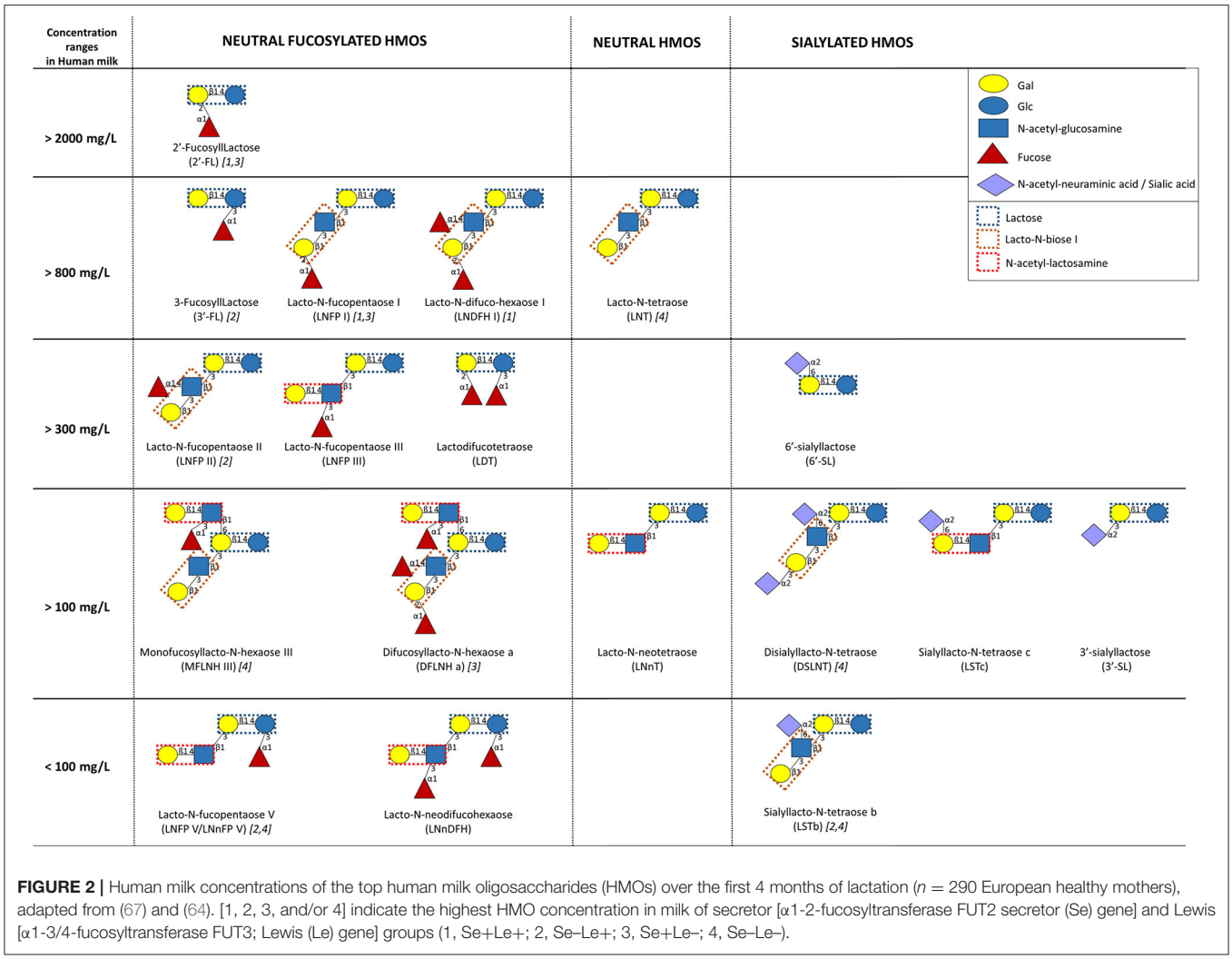

FIGURE 2 | Human milk concentrations of the top human milk oligosaccharides (HMOs) over the first 4 months of lactation ($n = 290$ European healthy mothers), adapted from (67) and (64). [1, 2, 3, and/or 4] indicate the highest HMO concentration in milk of secretor [α1-2-fucosyltransferase FUT2 secretor (Se) gene] and Lewis [α1-3/4-fucosyltransferase FUT3; Lewis (Le) gene] groups (1, Se+Le+; 2, Se–Le+; 3, Se+Le–; 4, Se–Le–).

triiodothyronine), parathyroid (parathormone, parathormone-related peptide, and calcitonin), steroid (estrogen, progesterone, and adrenal steroids), gut (insulin, ghrelin, and obestatin), and adipocyte (leptin, adiponectin, and resistin) hormones as well as growth factors [epidermal growth factor (EGF), nerve growth factor, insulin-like growth factor (IGF)-I and II, relaxin, and TGF-α and β] (13).

The presence of leptin (72, 73), ghrelin (74), and adiponectin (75) in HM has deserved great interest and has been extensively studied in the last 15 years due to their key role in regulating eating behavior and metabolism (76). Leptin is transferred from the maternal circulation to HM (72), and HM leptin concentration correlates with maternal plasma leptin concentration and maternal body mass index (BMI) (73). Leptin is also produced by mammary epithelial cells and secreted in milk fat globules (77, 78). The production of leptin in breast tissue might be regulated physiologically according to the nutritional state of the infant, as suggested by Dundar et al., who showed different leptin levels in maternal milk of small for gestational age (SGA), large for gestational age, or appropriate for gestational age (AGA) infants (79). Similarly, a remarkable decrease in leptin levels from colostrum to mature milk was also observed in mothers who delivered SGA infants and not in mothers who delivered AGA infants, which may contribute to early catch-up growth of SGA infants (80). Leptin concentrations are also higher in term milk compared with preterm milk (81, 82) even if some contradictory results exist (83). Similar to HM leptin, HM ghrelin comes from maternal plasma (74) and is likely synthesized and secreted from the breast (84). Adiponectin has been measured in skim milk at concentrations higher (more than ×40) than that of the other major adipokines leptin and ghrelin and correlated positively with maternal obesity (74, 75, 85). Adiponectin concentrations were higher in preterm HM compared with term HM (82). HM growth factors (IGF-I, IGF-II, EGF, and insulin) have also been extensively studied due to their gut trophic effects (86). IGF-I and EGF were particularly high in colostrum, while insulin seems to be provided at relatively constant level in colostrum, transitional milk, and mature milk in preterm milk, with their concentrations decreasing postpartum in term milk only, with no difference between term and preterm

milk insulin concentrations at delivery (87). In SGA infants, however, a decrease in insulin level from colostrum to mature milk was reported (80). Insulin content in HM is directly in relation with its actual concentration in maternal blood (88).

Bacteria

Although HM, like other human fluids, has long been considered sterile, microorganisms have emerged as a natural part of HM [for a detailed review on milk microbiota composition and origin, please refer to dedicated reviews (89, 90)]. The first studies focused on the presence of bacteria during intra-mammary infections and the transmission of pathogens through breastfeeding (91, 92). The presence of a complex microbial moiety consisting of commensal bacteria associated with healthy HM is now widely accepted, at least once milk is expressed. Whether a complex and living microbial community can be associated with milk inside the breast and the mammary ducts remains to be determined. The presence of a complex microbial moiety in HM, hereafter referred to as "milk microbiota," is supported by numerous studies, especially in the last decade, through the use of high-throughput sequencing approaches (89, 90, 93–104) but also culture-dependent analyses (96, 105–107). Some studies considered bacteria isolated from HM as contaminants originating from mother skin and infant oral cavity (11); others suggest that HM bacteria partly originate from maternal gut through a yet-hypothetical entero-mammary pathway. Several questions remain on this complex microbial moiety of HM, in relation to its origin, the factors shaping its composition, its viability, and on its contribution to the establishment of the gut microbiota and subsequent health outcomes in infant.

Characterization of HM microbiota relies on different types of approaches, including culture-dependent and culture-independent approaches such as metataxonomics, based on 16S rRNA gene amplicon sequencing (89). Milk is generally collected after cleaning the breast, by manual expression or using a pump, although some studies also chose to collect milk in a non-aseptic environment and to characterize the "breastfeeding-associated microbiota of HM," as it is transmitted to infants (103). Methods used to explore HM microbiota are likely to introduce major differences in its composition between studies. Culture-dependent approaches will allow the identification of a fraction of viable bacteria, i.e., those who are cultivable in the growth conditions used, whereas culture-independent approaches will detect DNA of the total bacterial population, independently of their physiological state. Most of the latest approaches rely on amplicon sequencing targeting, mostly the bacterial fraction of microbiota and to lesser extent the fungal community. A few studies based on shotgun metagenomic approaches are now available, giving access to archaeal, fungal, and viral communities and to a prediction of functions of these bacteria (108–110). Besides, within molecular approaches, several technical factors related to sample preparation, sequencing platform, or analytical pipelines may introduce some variability (111–113). Due to the low HM microbial load and the use of PCR-based techniques, these molecular approaches are subject to environmental contaminations during sampling or sample processing, notably by kit reagents, as was established for the "placenta microbiota" (114, 115). The inclusion of negative controls ("reagent only") is thus important to determine background contamination and ensure subsequent removal of "contaminant reads." Despite all these sources of variations, the large number of studies has allowed a better characterization and understanding of this complex microbial moiety.

HM microbiota is characterized by a low bacterial load but a high diversity. The total bacterial load was evaluated to be $\sim 10^3$–10^4 cfu/ml (range 10^1–10^6) in healthy HM by numeration on non-selective media, depending on the media used or the collection mode (manual expression *vs.* pump) and $\sim 10^5$–10^6 cfu/ml by qPCR on total DNA (96, 105, 106, 116). This observation suggests that a part of HM microbiota is either non-viable or non-cultivable. Of note, these bacterial cells were mostly shown to exist in HM in a free-living state and not to be associated with human cells (116). Despite this low bacterial load as compared with the well-characterized gut microbiota, HM was found to harbor a complex and diverse microbiota with several dozens of genera and more than 200 species identified so far (90, 104, 106, 117, 118).

Among the most frequently cited taxa, *Staphylococcus* and *Streptococcus* have been identified as universally predominant in HM (97). Several additional taxa have been frequently cited, including *Corynebacterium*, *Bifidobacterium*, *Propionibacterium*, *Bacteroides*, *Enterococcus*, *Faecalibacterium*, *Lactobacillus*, *Veillonella*, *Serratia*, *Ralstonia*, *Acinetobacter*, *Rothia*, and several members of the Lachnospiraceae and Ruminococcaceae families, suggesting the existence of a core HM microbiota (85, 89, 91, 100, 103) (**Figure 3**). *Pseudomonas* has also frequently been proposed to be part of HM microbiota, although its presence may be attributed to contamination issues (106).

A cross-species analysis of milk microbiota even suggested that some of these frequently cited taxa could be universally shared within species, thus constituting an inter-species core milk microbiota (89). HM microbiota composition was globally confirmed by culture-dependent studies, albeit with overrepresentation of easily cultivable aerobic or aerotolerant members such as *Staphylococcus*, *Streptococcus*, and *Propionibacterium* (96, 105, 106). In a study of 31 HM, the combination of cultivation with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry identification allowed the identification of more than 1,000 colonies (106). In addition to *Staphylococcus* and *Streptococcus* isolates, which were dominant in all HM samples, other highly abundant genera, present in >50% of the samples, belonged to *Acinetobacter*, *Gemella*, *Rothia*, *Corynebacterium*, *Veillonella*, *Lactobacillus*, *Enhydrobacter*, and *Propionibacterium* (**Figure 3**). Isolation of obligate anaerobic species such as strict anaerobic *Bacteroidetes* or *Clostridium* members was improved following milk storage for 6 days at 4°C, suggesting that these taxa also belong to the viable fraction of milk microbiota despite poor retrieval up to now (105). The fungal and viral HM communities have also started to be explored (122–124). The presence of fungi including *Saccharomyces* species has been reported in HM samples (122).

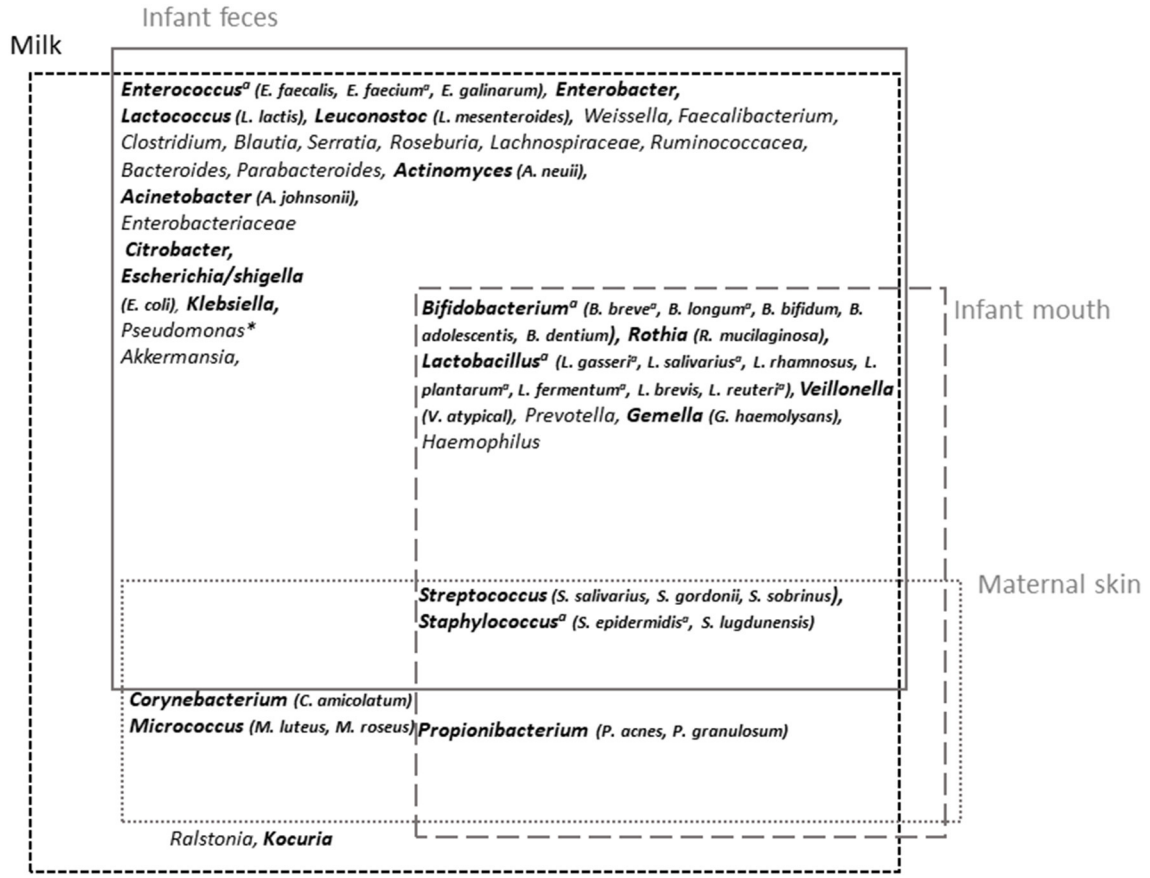

FIGURE 3 | Milk microbiota major taxa and overlap with infant feces and mouth and maternal skin microbiota. Overview of milk microbiota major taxa and their possible origin [based on the reviews by Oikonomou et al. (89) and Jost et al. (90) for milk microbiota and Byrd et al. (119) and Xiao et al. (120) for maternal skin and infant oral microbiota, respectively, and based on comparative studies between microbiota associated with these different sites (98–100, 106, 121)]. The taxa in bold correspond to those for which isolates have been obtained (∼viable fraction of milk microbiota). [a]Taxa for which shared strains between milk and infant feces have been reported; *generally considered as a contaminant.

The origin of HM microbiota remains a matter of debate. HM microbiota is likely a combination of microorganisms originating from maternal skin or even mammary tissue (125) and infant oral cavity. It may also result from the maternal digestive tract through a yet-hypothetical entero-mammary pathway involving immune cells (90). In agreement with the two former sources, *Propionibacterium* sp., *Staphylococcus* sp., or *Corynebacterium* sp. are usual members of the adult skin microbiota, and the presence of several members of the infant oral cavity in HM has been reported (104, 118). The infant oral microbiota contribution to HM microbiota was evaluated to be ∼21 and 66% 2 days and 5 months after birth, respectively (101). In agreement with the inoculation of HM microbiota by the infant mouth through a retrograde flow back into the mammary duct during suckling, Biagi et al. reported enrichment of HM microbiota by typical oral bacteria such as *Streptococcus* and *Rothia*, after the infant latching to the mother's breast, compared with HM microbiota collected by pump (99). Kordy et al. (126) also reported maternal areolar skin and infant oral cavity as major source of the breast milk microbiota, with an average contribution of 46 and 26%, respectively. Albeit controversial, the existence of an entero-mammary pathway has been proposed (127). This route is supported by a partial overlap between maternal feces and HM microbiota compositions (101, 128). In a study comparing the milk, vaginal, and fecal microbiota, Avershina reported a low redundancy in terms of bacterial species between these three microbiota, but HM had higher intra- than inter-individual similarities toward both vaginal and stool samples, supporting, to a certain extent, the translocation of gut microbiota to the milk (128). Using shotgun metagenomic sequencing, Kordi et al. identified the same strain of *Bifidobacterium breve* in maternal rectum, breast milk, and the stool of an infant delivered via caesarian section, suggesting direct transmission from maternal gut (126). The existence of the endogenous route is also supported by the isolation of common strains of *Bifidobacterium longum* from maternal and neonatal feces as well as from HM (46). Additionally, oral administration of some lactobacilli strains to lactating women led to their presence in milk (129, 130). This entero-mammary pathway may account for the presence of DNA corresponding to major gut-associated obligate anaerobes,

including *Bacteroides*, *Prevotella*, *Blautia*, *Clostridium*, *Dorea*, *Eubacterium*, *Coprococcus*, *Faecalibacterium*, or *Roseburia* (90).

Several factors have been proposed to shape HM microbiota composition. Strong inter-individual variations may be the result of both mother-related and environmental factors (89, 102, 104, 131). HM microbiota changes with time and notably between colostrum and mature milk (104, 116, 118). Differences were also reported in relation to the delivery mode, BMI, and parity (102, 104, 132–134). However, depending on the study design or methods used, contradictory results reporting the lack of effect of most of these factors have also been proposed, including the lactation stage (102), the delivery mode (caesarian section vs. vaginal delivery), the gestation length (preterm *vs.* term) (135), or the mother's BMI (136). The mode of breastfeeding itself was shown to affect HM microbiota. Pumped HM was associated with a higher abundance of potential pathogens and lower abundance of bifidobacteria and oral cavity-related species (99, 106, 134). Finally, HM microbiota is clearly affected by the mother health status including mastitis development and antibiotherapy or chemotherapy, which can directly affect microbial diversities and profiles (89, 108, 137–139).

Metabolites and Bacterial Metabolites

HM also contains many small molecules (<1,500 Da), which have recently deserved much interest due to their potential role on infant growth as well as on the development of the gut, immune, and nervous systems and other tissues (140). These small molecules include molecules found in milk fat globules (triacylglycerol species, glycerophospholipid species, sphingomyelin species, cholesterol, etc.) as well as proteins and peptides (proteinogenic amino acids), disaccharides and oligosaccharides (glucose, galactose, fucose, etc.), and other components dissolved in HM (amino acids, creatinine, urea, citrate, 2-keto-glutarate, choline, nucleotides, polyamines, etc.) (140). They have been identified using metabolomics methodologies (nuclear magnetic resonance spectroscopy or mass spectrometry); and depending on the technique and HM sample preparation, from hundreds (141, 142) to more commonly dozens of metabolites have been described in HM (143, 144). HM metabolite concentrations change with the duration of lactation (143), notably carbohydrates and amino acids during the first month of lactation (140), with high levels of amino acids in colostrum and high levels of saturated acids and unsaturated acids in mature milk (145). There are also differences in preterm and term HM metabolite concentrations, mainly in early lactation (140, 146). HM metabolites differ across specific geographical locations (China *vs.* Finland vs. South Africa, for instance) (144, 147). The pathophysiologic status of the mother influences the metabolite content of HM. HM of women with irritable bowel syndrome displays less sugar metabolites (lactose) and 2-aminobutyrate and more energy metabolites (succinate and lactate) than HM of healthy mothers (148). Likewise, gestational diabetes mellitus is associated with alterations in the metabolome of HM, especially the colostrum (145).

HM metabolites may be filtered from the mother bloodstream through the mammary epithelium, may originate from different metabolic processes within the mammary gland, or may be produced through the metabolic processes of resident microbes in HM (149). It is difficult to ascertain the microbial origin of HM metabolites, as many metabolites can be produced by both bacteria and eukaryote cells, but some HM metabolites are more likely to be of bacterial origin. This is the case of biogenic amines including the polyamines (spermine, spermidine, and putrescine), together with the monoamines (tyramine) and diamines (histamine and cadaverine) (149). *Enterococcus*, a major bacteria group in HM, are the main producers of biogenic amines, mainly putrescine and tyramine (150). A positive correlation between putrescine concentration and *Pseudomonas fragi*, a Gammaproteobacteria, has recently been described in HM (151). HMOs could also be a direct substrate for HM bacteria, which would produce metabolites. However, to our knowledge, the correlation between specific products of HMO fermentation and HM bacterial strains has never been described. In a recent study, Mai et al. demonstrated *in vitro* that HM promoted the growth of probiotic *Lactobacillus reuteri* DSM 17938, a strain originally isolated from HM, and its secretion of potentially beneficial metabolites (such as succinate, glutamine, *N*-acetylcysteine, citrulline, spermidine, and lactate) (152), suggesting that HM could indeed favor the growth and metabolism of HM bacteria, generating specific bacterial metabolites.

ROLE OF THE DIFFERENT BREAST MILK COMPONENTS IN SHAPING THE INFANT GUT MICROBIOTA

Oligosaccharides

Among HM components, HMOs, which are both non-digestible molecules utilized by commensal infant bacteria in the large intestine and free competitor to enteric pathogens, are known to strongly influence the composition of the infant gut microbiota. Several studies have shown that the fecal bacterial composition of breastfed infants is different from that of formula-fed infants (64, 153). The fecal microbiota composition of formula-fed infants devoid of HMOs is poorer in bifidobacteria than that of breastfed infants. While the microbiota of breastfed infants was represented by 90% of bifidobacteria and lactobacilli, that of formula-fed infants was composed of 40–60% bifidobacteria and lactobacilli, and the remaining represented by Enterobacteriaceae and *Bacteroides*. In addition, the rate of establishment of gut microbiota in infants breastfed by secretor mothers is faster than in those breastfed by non-secretor mothers (64, 154). Furthermore, the microbiota composition of breastfed infants from non-secretor mothers was shown to be slightly different from the microbiota of infants breastfed by secretor mothers with higher colonization by *Bifidobacterium adolescentis* and absence of *Bifidobacterium catenulatum* (155). These latter observations demonstrated the major role of HMOs in the establishment of the infant gut microbiota.

The predominant members of the early gut microbiota, *Bifidobacterium*, *Bacteroides* spp., and *Lactobacilli*, possess the ability to utilize HMOs by fermentation, while other members, including *Clostridium*, *Enterococcus*, *Escherichia*,

Eubacterium, *Staphylococcus*, *Streptococcus*, and *Veillonella* spp., do not (64, 90, 156–159). *In vitro* analyses showed that the major *Bifidobacterium* strains (*Bifidobacterium breve and Bifidobacterium bifidum*) present in the infant gut microbiota were also the major strains able to ferment HMOs (159). Moreover, Borewicz et al. showed a relation between HMO consumption patterns and specific microbial groups affecting both bacteria possessing the ability to utilize HMOs and the others (160).

The prebiotic role of HMOs on the infant microbiota can be partly attributed to their specific structures. HMO consumption is mainly associated with *Bifidobacterium* genus but is also found in a few *Bacteroides* and *Lactobacillus* species. However, the ability to consume HMOs is not characteristic of all bifidobacterial isolates, and certain HMOs are more utilized by bifidobacteria than others (161). Moreover, cross-feeding between HMO degraders and non-HMO users has been observed (162). Genomics, transcriptomics, and glycobiology methods have been useful to study the molecular basis of this preferential utilization of HMOs by bifidobacteria species, especially the induction of specific genes in the presence of HMOs, which confer a selective advantage on this substrate (161). As a consequence of this preferential use of HMOs by some specific strains, analyses of the HMOs and the fecal microbiota composition of 1- and 3-month-old breastfed infants showed that 2′-FL and LNFP-I, which are the main oligosaccharides found in the milk of secretor mothers, affect the infant gut microbiota (160, 163). Among the synthetized HMOs, 2′-FL and lacto-*N*-neotetraose (LNnT) are widely studied and are considered safe for infant nutrition. Fecal microbiota composition of 2′-FL- and LNnT-supplemented formula-fed infants was more similar to that of breastfed infants, in terms of microbial diversity, global composition at the genus level, and abundance of several major genera than that of infants fed a non-supplemented formula (64, 164, 165). Moreover, 2′-FL and LNnT supplementation was associated with lower prescription of antibiotics during the first year of life, although fecal microbiota profiles no longer differed between supplemented and non-supplemented infants at 12 months of age (165). Likewise, sialic acid is known to be an essential nutrient during periods of rapid neural growth and brain development in the newborn (166). α2-6-Linked sialylated oligosaccharides were present in greater proportion than the α2-3-linked structures during early lactation (167, 168). Recently, Bondue et al. demonstrated the ability of a specific Bifidobacteria, *Bifidobacterium mongoliense*, to utilize 3′-SL as the main source of carbon (169).

Individual or mixed HMOs also have a preventive role in the attachment of pathogens in the infant gut. Some HMOs mimic lectins or glycan-binding proteins, preventing pathogen attachment on epithelial surfaces. 2′-FL was reported to alleviate inflammation, lower allergic reaction, and prevent enteric pathogens (such as *Campylobacter jejuni* or *Escherichia coli*) attachment on epithelial surfaces (170, 171). α1-2-Fucosylated HMOs act as antiadhesive antimicrobials against *C. jejuni* (170, 171). For some pathogens such as *Entamoeba histolytica*, complex HMOs containing Gal/GlcNAc patterns (LNFP II and LNFP III but not LNFP I, which contain α1-2-fucose residue) are required to block attachment or cytotoxicity (172). Interestingly, 2′-FL and 6′-SL were found to directly bind to TLR4 and inhibit TLR4 signaling in *ex vivo* gut tissue and organoid cultures, explaining the protection against the necrotizing enterocolitis in newborn mice and premature piglets (173). Recently, Wang and collaborators demonstrated in mice that 2′-FL intake increased the abundance of *Akkermansia* spp., a probiotic potentially involved in the expression of mucins in goblet cells and thus the reduction of the colonization of the harmful bacteria *E. coli* O157 (174).

Milk Bacteria

Considering an estimated daily ingestion of log 5 to 7 HM-associated bacteria, HM microbiota is a continuous source of commensal or probiotic microbes able to colonize the gut or influence the infant gut microbiota during the first stage of life (90, 101, 118). Strong overlap exists between milk and the infant gut microbiota when considering major taxa of milk microbiota (**Figure 3**). Several studies intended to evaluate the role of HM microbiota in the infant gut colonization, revealing some discrepancies between them due to both the methods and the taxonomic levels used to compare microbiota. A strong overlap between the infant gut and milk microbiota was pointed out by Pärnänen et al. in a metagenomic analysis, as 76% of the species found in milk were present in the infant gut (110). In this study, a strong overlap was also revealed for antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs) between milk and infant feces, as 70% of the ARGs detected in milk were present in infant feces. Conversely, infant feces shared 20% of their ARGs and 12% of their MGEs with HM. William et al. estimated a direct contribution of only 4.9% of HM microbiota to the infant gut microbiota and suggested indirect contribution through an effect on microbiota in the upper part of digestive tract, including the oral microbiota (101). Despite this low direct contribution of HM microbiota to the infant gut microbiota, these two communities were found to be intimately linked as revealed by correlation analyses (101). Using a similar tool (i.e., SourceTracker), Pannaraj et al. estimated the proportion of bacteria in infant stool originating from HM to be 27.7 and 10.4% for primarily breastfed and non-primarily breastfed infants in the first month of life, and this contribution decreased thereafter (175). In agreement with the role of HM microbiota in shaping the infant gut microbiota, the infant gut microbiota and even the resistome were more similar to each infant own mother's gut microbiota than to unrelated women (99, 110). Likewise, Biagi et al. (99) investigated the relation between HM, oral microbiota, and fecal microbiota in preterm infants whose breastfeeding mode changed from indirect intake through breast pump to direct breastfeeding. A non-supervised approach allowed defining three HM bacteria community types that were more or less related to the breastfeeding mode. Interestingly, compositional differences between these milk community types were associated with compositional differences in infant fecal and oral microbiota. Similar conclusions were supported by a study based on nearly 400 mother–infant dyads in 11 international sites (100). In this study, despite limited associations between individual genera in

HM and fecal microbiota, community-level analyses suggested strong, positive associations between these two microbiota. Similar conclusions were drawn regarding the viral communities, which were distinguishable between HM and infant feces, but with a significant number of shared viruses in HM and feces from all mother–infant dyads (109). Thus, although all these studies differ in their rates of overlap and contribution, depending on the methods used, the cohorts, and whether the reference is HM or infant feces, they support partial overlap between HM and infant gut microbiota and suggest that they are both positively linked.

Shared species between HM and infant gut include the pioneer genera initiating gut microbiota assembly (46). They include facultative anaerobes such as *Staphylococcus*, *Streptococcus*, *Lactobacillus*, *Propionibacterium*, *Enterococcus*, or *Escherichia* species that contribute to generate an anaerobic environment and favor the subsequent implantation of obligate anaerobes such as *Bifidobacterium*, *Bacteroides*, *Blautia*, or *Veillonella* species (90, 176). Several studies reported the vertical transfer of *Bifidobacterium* species, which are dominant in breastfed infant gut (96, 124, 177). Biagi et al. (98) characterized the composition of the oral and fecal microbiota of infant and that of HM microbiota in 36 healthy mother–infant pairs and reported a limited number of operational taxonomic units (OTUs) shared among the three microbiota that belonged to the *Bifidobacterium* genus, as well as specific *Streptococcus* and *Staphylococcus* OTUs. These *Streptococcus* and *Staphylococcus* OTUs were dominant in the infant mouth ecosystem as well, supporting the baby's mouth as a transition point between HM and infant gut, contributing to both infant gut and mother's milk duct colonization.

The use of 16S rRNA gene-based molecular approach to investigate vertical transfer may be limited and subject to criticisms. Others studies combining culture-dependent approaches with genotyping of isolates reported the presence of the same strains in infant feces and HM, supporting a vertical transfer of both facultative and strict anaerobes (46, 90, 124, 178). In particular, few studies reported the presence of shared strains of *B. breve* and *Bifidobacterium longum* in HM and infant feces (46, 107, 177). Exploration of the *Bifidobacterium* and bifidophage population in the maternal and infant feces and HM of 25 mother–infant pairs through the combination of molecular and culture-dependent approaches revealed that similar OTUs or strains as well as bifidophages were shared between these three types of samples within mother–infant pairs (124). Apart from *Bifidobacterium* species, the presence of shared strains belonging to *Staphylococcus*, *Enterococcus*, and *Lactobacillus* in HM and infant feces was also demonstrated through genotyping of isolates (**Figure 3**) (90, 107, 178–180). Martin et al. (177) notably reported the presence of two to four shared strains of *Staphylococcus*, *Lactobacillus*, and/or *Bifidobacterium* between HM and infant feces from 19 mother–infant pairs (177). Transfer of other strict anaerobes such as *Bacteroides* or *Veillonella* species still remains to be clearly demonstrated by culture-dependent methods. Additional studies based on high throughput culturomic approaches may help to evaluate the proportion of shared strains between HM and the infant gut microbiota.

An alternative to identify HM bacteria that are able to colonize the gut was proposed by Wang et al. (181). By inoculating normal chow-fed germ-free mice with HM, they reported the presence in the feces of OTUs belonging to *Streptococcus*, *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* genera as well as anaerobic gut-associated bacteria belonging to *Faecalibacterium*, *Prevotella*, *Roseburia*, *Ruminococcus*, and *Bacteroides* at low abundance. *Bifidobacterium* was also isolated from mice feces at very low abundance, although it was below the detection limit in HM (181). Of note, although some species were shared between HM and infant feces, their relative abundance within microbiota strongly differs. This is the case for *Bifidobacterium* whose abundance was low in HM but which became dominant in the infant gut, due to modifications of growth conditions and to their ability to metabolize HMOs (4, 90).

Beyond a direct role in seeding the infant microbiota, HM microbes likely contribute to gut microbiota assembly through their effects on gut microbes, including competition for nutrients or gut mucosal binding sites, direct inhibition, or contribution to trophic chains. Hence Jost et al. (90) suggested a role of HM bacteria in gut lactate metabolism. Most of HM bacteria are involved in either lactate production (*Staphylococcus*, *Streptococcus*, and *Lactobacillus*) or lactate utilization (*Propionibacterium* and *Veillonella*), which could favor the establishment of balanced metabolic activities in the gut and prevent disorders related to lactate accumulation but also influence gut microbiota establishment through this trophic chain. Regarding inhibition potential of HM microbiota, HM contains several bacteriocin-producing strains, such as *Enterococcus faecalis*, *Enterococcus faecium*, and *Staphylococcus* sp., that may provide them a competitive advantage in the colonization of the infant gut or contribute to shaping of the infant gut microbiota (182). Likewise, HM contains bacteriophages that are partly transmitted to the infant gut and that could influence the infant gut microbiota composition (109). Finally, HM microbiota may also influence the infant gut microbiota assembly through their effect on gut immune system via their immunomodulation properties (modulation of cytokine production and induction of SIgAs) or their impact on gut barrier function (183).

As previously mentioned, part of the HM microbiota is non-viable or, at least, non-cultivable. This was revealed by discrepancies between the total bacterial load of HM as determined by culture-dependent or metataxonomic approaches and differences in the microbial profile with overrepresentation of few genera in culture-dependent approaches (96, 105). However, strains corresponding to obligate anaerobes have been isolated (105), suggesting that the living part of HM microbiota is underestimated. Further exploration of HM microbiota using high-throughput culture-dependent methods is now required to fully understand the contribution of HM microbiota to the infant gut microbiota. Moreover, even if the living part of HM microbiota is underestimated, part of this microbiota is likely inactivated during the first steps of digestion. This raises questions about the role of this "non-living" part of the

microbiota, since bacterial antigens would still be able to interact with the host immune system and indirectly contribute to the shaping of infant gut microbiota.

Immune Factors

Lactoferrin

LF has a direct cytotoxic effect against a large panel of microorganisms (bacteria, viruses, and fungi), mainly in the gut mucosa. For example, the iron-free form of LF can kill *Streptococcus mutans*, *Streptococcus pneumoniae*, *E. coli*, *Vibrio cholera*, *Pseudomonas aeruginosa*, and the fungal pathogen *Candida albicans* (184). Moreover, LF also has bacteriostatic properties due, in part, to its ability to bind ferric ions and most of the iron from HM, thus reducing iron availability for bacteria. Multiple clinical studies have suggested a number of potentially favorable biologic effects associated with LF in infants and children. The first randomized controlled trial assessing LF supplementation in neonates reported a reduction in the incidence of late-onset sepsis in bovine LF supplemented compared with placebo in preterm infants (185). Data from the recently completed ELFIN (enteral LF in neonates; $N = 2{,}200$) and LIFT (LF infant feeding trial; $N = 1{,}500$) studies will help clarify the potential benefits of LF supplementation in preterm infants (186). Moreover, fragments of human LF and of pIgR stimulate the growth of a large set of *Bifidobacterium* strains. Indeed, the fragments of LF and pIgR are 100 times more effective to enhance *Bifidobacterium* growth on a molar basis than the carbohydrate *N*-acetyl glucosamine, a currently known bifidogenic carbohydrate, leading to the assumption that the bifidogenic activity of HM based on peptides exceeds that of some HM carbohydrates (187).

Secretory IgA

In suckling infants, SIgAs shape the composition of the gut microbiota. Immune exclusion is one of the most commonly proposed mechanisms by which SIgAs block microbes from attaching to, colonizing, and invading mucosal epithelial cells. Indeed, SIgAs in HM inhibit the binding of *Clostridium difficile* toxin A to enterocyte brush border membrane receptors (188). Moreover, secretory component (SC) alone is sufficient to inhibit toxin binding to receptors. SC is primarily responsible for blocking toxin A attachment to epithelial cell monolayers. Furthermore, SC may serve as a decoy receptor for other pathogens, including entero-toxigenic *E. coli* (189).

IgG

The presence of IgG in HM helps in counteracting the infant deficiencies in opsonization and antibody-mediated cytotoxicity. Antibodies that recognize antigens expressed by entero-toxigenic *E. coli* and other Enterobacteriaceae species of the maternal microbiota are produced and secreted in HM (190). IgG is also important for establishing homeostasis with regard to the newly colonizing microbiota by prevention of the activation of the gut-associated lymphoid tissue (191).

Lysozyme

Lysozyme, also called *N*-acetyl muramidase, hydrolyses peptidoglycan polymers of bacterial cell walls at the β1-4 bonds between *N*-acetyl muramic acid and *N*-acetyl glucosamine, thereby lysing Gram-positive bacteria. *In vitro* study using electron microscopy demonstrated that lysozyme can act synergistically with LF to help in bacterial clearance (192). LF first binds to the lipopolysaccharides of the outer cell membrane of the Gram-negative bacteria, creating holes in the membrane. Lysozyme can then enter and degrade the peptidoglycan of the bacteria, killing the pathogens (192).

Cytokines

Cytokines participate in the establishment and maintenance of tolerance to harmless food antigens and commensal bacteria (53). However, their precise role in shaping the infant gut microbiota still needs to be demonstrated.

Although many data obtained *in vitro* indicate a possible effect of HM immune factors in modulating the infant gut microbiota, this effect is not supported yet by clinical or animal model studies, except for LF. Further studies are therefore needed to fully assess their role.

Bacterial Metabolites

It is difficult to speculate on the role of HM bacterial metabolites, as, as seen earlier, the specific bacterial origin of HM metabolites is still difficult to ascertain. Polyamines are mainly bacterial end products and do not interfere with bacterial growth. Thus, HM polyamine content is unlikely to modulate the infant gut microbiota. If lactate, short-chain fatty acids, and intermediary metabolites such as succinate, are effectively produced by HM bacteria and released in HM, then a differential production of these metabolites could interfere with infant microbiota, but such a direct link is speculative, and further studies on the specific role of HM bacterial metabolites on shaping of the infant gut microbiota are warranted.

Macronutrients

Lipids

The role of HM lipid fraction on the infant gut microbiota is poorly documented, but several lines of evidence point to a possible effect. Indeed, *in vitro* studies reported either bactericidal activities of milk lipids, including medium-chain fatty acids (MCFAs), sphingosine, and monoacylglycerols (193), or a bacterial growth promotion activity, especially a beneficial effect of oleic acid on *Lactobacillus* species (194). Accordingly, Nejrup et al. observed significant changes in infant fecal microbial communities (increased *Lactobacillus* and *Bifidobacterium* abundances and decreased Enterobacteriaceae abundance) cultured with selected HM lipids MCFA, monoacylglycerol, and/or sphingosine during anaerobic *in vitro* fermentation (195). Investigations of the effect of lipid fractions of infant formulas on the infant gut microbiota or of associations between HM lipid fractions and infant microbiota composition are also available. Increasing the proportion of palmitic acid in the sn-2 position of triglycerides in infant formula increased fecal *Lactobacillus* and *Bifidobacterium* counts after 6 weeks (196).

A significant association between the proportion of decanoic acid, myristic acid, stearic acid, palmitic acid, arachidonic acid, and docosahexaenoic acid in the sn-2 position of triglycerides in HM of Chinese women and *Bacteroides*, Enterobacteriaceae, *Veillonella*, *Streptococcus*, and *Clostridium* abundance of their infant gut microbiota has recently been described (197). HM gangliosides could also participate in the shaping of the infant gut microbiota. They are glycosphingolipids consisting of a hydrophobic ceramide and a hydrophilic oligosaccharide chain and have been described as putative decoys that interfere with pathogenic binding. Infant formula enriched in ganglioside reduced *E. coli* counts and slightly increased bifidobacteria counts (+0.5 log/g feces) in preterm infant feces after 30 days (198). Likewise, the HM sphingolipids could affect the gut microbiota since several reports in mice indicated an effect of dietary bovine sphingolipids on microbiota composition (199). Yet their effect on the infant gut microbiota has not been investigated to our knowledge. Finally, addition of milk-fat globule membranes to formulas in neonatal piglets shifted their fecal microbiota toward the composition of sow-reared piglets as opposed to plant lipid-based formula (24), with similar results in rats (200).

Carbohydrates

Lactose

Beside HMOs, whose role in shaping the infant gut microbiota has been discussed above, HM lactose could also contribute, yet to a lesser extent, to the infant gut microbiota establishment. Although large amounts of lactose are unlikely to reach the large intestine due to its hydrolysis and absorption within the small intestine, lactose is easily degraded by several bacterial species (201–203). An association between lactose concentration and the colonic microbiota composition in formula-fed piglets has been described (204). *In vitro* data also suggest a synergy between lactose and oligosaccharides on *B. longum* growth (205). Similarly to lipids, the data on the role of lactose on the infant microbiota are scarce. Consumption of a lactose-reduced and added-sugar (corn-syrup solids) formula by infants for 6 months slightly increased the diversity (+18%) and Acidaminococcaceae abundance (+0.7 log/mg feces) in feces compared with lactose-containing formula consumption (206). These effects were not reproduced in a preterm piglet model where diversity was lower in corn-syrup solid formula-fed piglets compared with lactose formula-fed ones (207).

N-Glycans

The role of N-glycans in shaping the infant gut microbiota has been highlighted in a piglet study evaluating the postnatal concentration of N-glycans in sow milk and the piglet microbiota composition in parallel. This study indicated that milk N-glycome correlated to abundances of certain gut microbes, either positively or negatively (208). However, data on correlations between human infant gut microbiota and HM N-glycans are not available yet to our knowledge. The enzymatic equipment and catabolic pathways to use these N-glycans have been identified in certain isolates of commensal *Bifidobacterium* (209) and *Lactobacillus* (210). Some infant-borne bifidobacteria such as *B. longum* subsp. *infantis* were found to harbor a cell-wall associated endo-β-*N*-acetylglucosaminidase able to release oligosaccharides from milk proteins (209). These milk glycoprotein-derived oligosaccharides can serve as selective substrates for the growth of these infant-associated bifidobacteria, similar to HMO (211). However, to our knowledge, data on correlations between human infant gut microbiota composition and HM N-glycans are not available.

Proteins and Non-protein Nitrogen

The impact of HM proteins and NPN compounds on the infant gut microbiota has been suggested for proteins and peptides with immunomodulatory properties (described above). Yet other HM proteins and NPN compounds could also affect the infant gut microbiota composition. Several animal studies evaluated the impact of whey protein content in formula on gut microbiota composition. Colonic microbiota diversity and relative abundances of Clostridiaceae, Enterobacteriaceae, *Streptococcus*, and *Streptomyces* were increased in preterm piglets receiving a formula with α-lactalbumin-enriched whey protein concentrate for 19 days (212). However, this was not reproduced in term infants since a formula enriched in α-lactalbumin and glycomacropeptides did not affect fecal microbiota composition in 6-month-old term infants (213). Likewise, a whey or whey-and-casein formula did not affect the gut microbiota in preterm piglets (214). HM mucins may also affect the gut microbiota implantation or at least protect from pathogens. Indeed, mucins have been shown to inhibit some pathogens like rotavirus by inhibiting its replication (215, 216) or *Salmonella enterica* serovar Typhimurium by inhibiting its binding properties on host cells (217).

Although whey protein and casein do not seem to be major HM components in orientating gut microbiota composition, other data support a role of NPN compounds. Indeed, recent work by the Sela group indicated that several *B. infantis* strains were competent for urea nitrogen utilization and that urease gene expression and downstream nitrogen metabolism pathways were induced during NPN utilization (218). Nucleotides may also drive the gut microbiota development since a nucleotide-enriched formula was shown to reduce the *Bacteroides–Porphyromonas–Prevotella* group to *Bifidobacterium* species ratio in the feces of 20-week-old healthy infants, compared with standard formula (219).

Hormones

HM hormones retain their biological activity in the infant gut, possibly due to post-transcriptional modification in the mammary gland before secretion into HM, which may increase their resistance to digestion (13). If their role in favoring proliferation of intestinal cells, increasing mucosal growth, enterocyte migration rates, villus height, brush border enzymes activity, and expression of glucose transporters (220) as well as their effects on metabolism through their absorption in infant plasma (76) is well documented, their role as contributors to the colonization patterns of the infant gut microbiome is much less documented. A recent study by Lemas et al. in 2-week-old exclusively breastfed infants

highlighted a positive association between HM insulin and both microbial taxonomic diversity and Gammaproteobacteria abundance (e.g., Enterobacteriaceae), whereas HM insulin was negatively associated with Lactobacillales abundance (e.g., Streptococcaceae) (221). As suggested by the authors, this may be due to a direct role of insulin to regulate enterocyte maturation and/or the ability of oral insulin to increase glucose concentration in the gut lumen. As Enterobacteriaceae are a family of glucose metabolizers, their gut colonization could therefore be favored.

In the same study, metagenomic analysis showed that HM leptin and insulin were associated with decreased bacterial proteases implicated in gut permeability and reduced concentration of pyruvate kinase, a biomarker of pediatric gut inflammation (221). There was no association between HM leptin and microbiota (221) even if a role of leptin in modulating gut microbial composition has been suggested in rodent; but this effect, mediated by differential expression of the mRNA expression of gut antimicrobial peptides, did not imply gut leptin receptors (222). However, it has recently been evidenced in rats that supplementation during the first 21 days of life with leptin or adiponectin decreased the abundance of the Proteobacteria phylum and the presence of *Blautia* (223). Moreover, leptin-supplemented rats had lower relative abundance of *Sutterella* and a higher proportion of *Clostridium* genus, among others. Supplementation with adiponectin resulted in lower abundance of the *Roseburia* genus and a higher proportion of the *Enterococcus* genus (223). Oral insulin may also have an antimicrobial action against potential pathogens through upregulation of a specific endotoxin receptor on the gut brush border membrane, as demonstrated in suckling mice receiving insulin orally every day (224).

IMPACT OF MATERNAL DIET ON MILK COMPOSITION: A NUTRITIONAL STRATEGY TO SHAPE THE INFANT GUT MICROBIOTA ASSEMBLY

The impact of maternal diet upon HM macronutrient, micronutrients, and immune factors has been reviewed recently (20). But it is not presented here, so to concentrate on the impact of maternal diet on HMOs, bacteria, hormones, and bacterial metabolites, which are less documented.

Human Milk Oligosaccharides

Besides genetic factors presented above, physiological and environmental factors such as maternal nutritional status, geographical origin, or type of delivery were shown to affect the composition and amount of HMOs. However, only few studies investigated the impact of maternal diet on HMO composition. In extreme environmental conditions, maternal nutritional status, due to seasonal fluctuations that affect food reserves and diversity in Gambia, was shown to affect the amount of HMOs. Gambian mothers ($n = 12$) who gave birth during the wet season where food is highly depleted had a lower amount of HMOs than mothers who gave birth during the dry season ($n = 21$) (61). An effect of high protein or fiber content in maternal diet during pregnancy and lactation was also suggested in a rat study, with an increase of a neutral oligosaccharide and an acidic oligosaccharide among identified oligosaccharides (225). A recent study observed the presence of the diet-derived sialic acid Neu5Gc in HMOs in 16 samples of HM (226). Because of the human inability to synthesize Neu5Gc, its presence in HM is a clear evidence of a direct influence of maternal diet on HMO biosynthesis, although the positive association observed between ingested and observed (in HMOs) Neu5Gc levels was not significant. In addition, total fruit intake and cured meat intake, positively and negatively, respectively, correlated with the abundance of several HMOs, while cheese intake positively correlated with Neu5Gc levels (226). Moreover, a preliminary study showed that lower BMI (14–18 compared with 24–28) correlates with a lower amount of HMOs (62). Since then, several studies confirmed that pre-pregnancy BMI impacts the composition and the concentrations of HMOs during the first 4 months of lactation (67, 227, 228). Negative associations of maternal fat mass with fucosylated HMOs were also highlighted, reinforcing the role of maternal nutritional status before and during pregnancy on the composition of HMOs (172, 229). Finally, a recent interventional study using a crossover design in two different cohorts tested the effect of glucose or galactose-enriched diet for 30–57 h ($n = 7$) or a high-fat or high-carbohydrate diets for 8 days ($n = 7$) with 1–2 weeks washout between diets. Interestingly, HMO-bound fucose concentration was reduced with the glucose-enriched diet, while HMO-bound sialic acid was reduced with the high-fat diet (230). Although the cohort was relatively small and dietary intervention short, this type of clinical interventional studies with a crossover design would be insightful to fully assess the role of maternal diet upon HMO composition.

Milk Bacteria

Factors such as geography have been shown to play a role in HM microbiota composition (144, 231, 232), although several factors may be indirectly responsible for these geographical differences, including lifestyle, environment, or diet. The relationship between maternal diet and HM microbiota was indirectly reported by Kumar et al. (231), who established a correlation between HM microbiota and specific fatty acid profiles. Likewise, HM microbiota composition was related to fatty acids, carbohydrates, and protein intake as observed by Williams et al. (102). However, the impact of maternal diet on HM bacteria deserves further investigations. Seferovic et al. in their crossover study investigating the impact of glucose or galactose on the one hand and of high-fat vs. high-carbohydrate diets in two small cohorts revealed overall minimal discernable impact of maternal diet on taxonomic composition of HM (shotgun metagenomic sequencing). However, the abundance of multiple metabolic pathways was influenced by maternal diets, including pathways involved in amino acid metabolism (230). Once again, well-powered and long-duration intervention clinical trials are warranted to further explore the role of maternal diet upon HM microbiota.

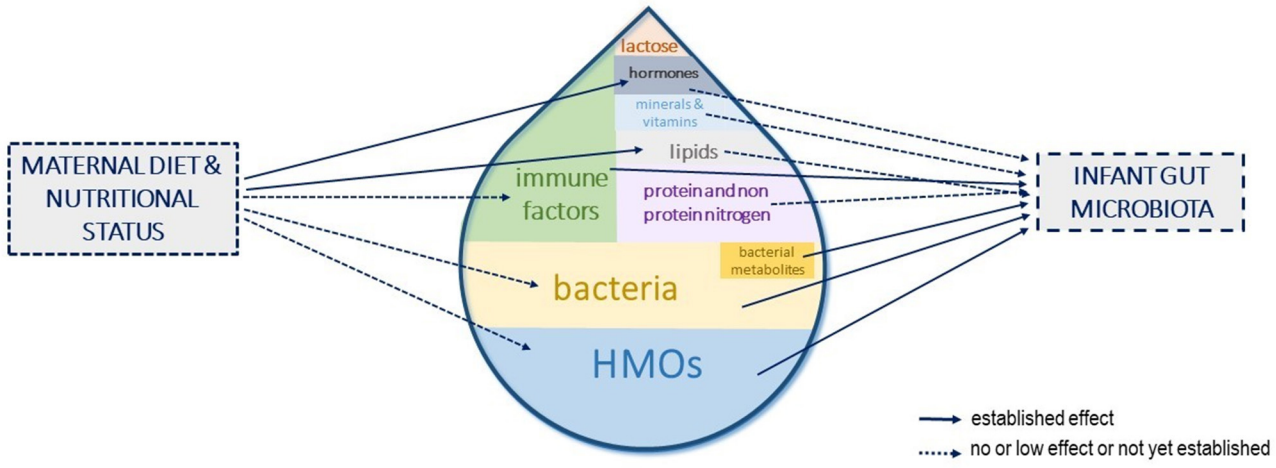

FIGURE 4 | Human milk components shaping the infant gut microbiota and influence of maternal diet and nutritional status. The different components of human milk (HM) that have been shown or are suspected to influence the infant gut microbiota establishment are represented. Their size in the milk drop is proportional to their suspected role in shaping the infant gut microbiota. The influence of maternal diet or nutritional status on these HM components concentrations is depicted with arrows.

Bacterial Metabolites

The impact of maternal diet on HM metabolites has been indirectly studied through their characterization in HM from different geographical (and therefore different diets) locations (cf. *Metabolites and Bacterial Metabolites* section) and comparing HM metabolites in lean and obese mothers (229). At 1 month postpartum, 10 HM metabolites differed between overweight/obese and lean mothers: 4/10 metabolites were nucleotide derivatives, 3/10 were HMOs, and one was a butyrate derivative (2-aminobutyrate) (229). In another study, the total polyamine content was lower at 3 days, 1, and 2 months after delivery in HM from obese mothers compared with HM from lean mothers (233). Spermine levels did not differ between groups at any time in contrast to the levels of putrescine and spermidine. The obese mothers who received dietary advice during pregnancy based on the Nordic Nutrition Recommendations had higher concentrations of putrescine and spermidine in their milk than the obese mothers without any intervention, suggesting that the low levels in obesity were at least partly associated with food habits. However, the consistency of spermine suggested a special metabolic function of this polyamine (233). Finally, a choline supplementation during the second half of gestation and the first month and a half of lactation increased HM choline and its derivatives' concentration (234).

Hormones

As discussed earlier, HM hormones arise from maternal plasma. Thus, HM hormone concentrations are directly linked to maternal plasma concentrations (13) and thus maternal nutritional status. A direct role of maternal diet on HM hormone concentration is unknown.

CONCLUSION

HM is not only a biofluid that provides the nutrients required to promote infant growth. It also contains many components whose impact on the infant gut microbiota establishment starts to be recognized. Data on causal relationships between these compounds and the infant microbiota are scarce. Current evidences rely on *in vitro* data, animal models, or association studies in humans, which highlights the need for strong convincing studies. Moreover, the amount of HM compounds reaching the colon, the role of partly digested compounds (for example, HM-derived peptides) reaching the colon, and the role of intact HM compounds on small intestine microbiota composition also need to be investigated to fully appreciate the role of HM in shaping the infant gut microbiota.

At the maternal level, a better understanding of the factors influencing compounds' concentration in HM, the interactions between them, and the persistence of the effects could open avenues to strategies to modulate the infant gut microbiota toward compositions beneficial to their health. Among the influencing factors, lifestyle and diets could be used to shape HM components toward a targeted composition that could, in turn, shape the infant gut microbiota and more largely be beneficial to infant health (**Figure 4**). However, studies investigating the role of maternal diet upon the main contributors to the infant gut microbiota (i.e., HMOs, bacteria, and immune factors) are still lacking. Interventional trials in large cohorts with long dietary interventions, covering both gestation and lactation and/or observational studies with well-designed frequency food questionnaires to get an in-depth characterization of mothers' eating profiles, are needed to fully understand and use the maternal diet as a leverage to shape the infant gut microbiota.

AUTHOR CONTRIBUTIONS

GB, EC, IL, SF-B, SL, SE, and SB wrote the manuscript. GB coordinated the review. All authors approved the final version of the manuscript.

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Safety and Modulatory Effects of Humanized Galacto-Oligosaccharides on the Gut Microbiome

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Complex dietary carbohydrate structures including β(1–4) galacto-oligosaccharides (GOS) are resistant to digestion in the upper gastrointestinal (GI) tract and arrive intact to the colon where they benefit the host by selectively stimulating microbial growth. Studies have reported the beneficial impact of GOS (alone or in combination with other prebiotics) by serving as metabolic substrates for modulating the assembly of the infant gut microbiome while reducing GI infections. N-Acetyl-D-lactosamine (LacNAc, Galβ1,4GlcNAc) is found in breast milk as a free disaccharide. This compound is also found as a component of human milk oligosaccharides (HMOs), which have repeating and variably branched lactose and/or LacNAc units, often attached to sialic acid and fucose monosaccharides. Human glycosyl-hydrolases do not degrade most HMOs, indicating that these structures have evolved as natural prebiotics to drive the proper assembly of the infant healthy gut microbiota. Here, we sought to develop a novel enzymatic method for generating LacNAc-enriched GOS, which we refer to as humanized GOS (hGOS). We showed that the membrane-bound β-hexosyl transferase (rBHT) from *Hamamotoa (Sporobolomyces) singularis* was able to generate GOS and hGOS from lactose and N-Acetyl-glucosamine (GlcNAc). The enzyme catalyzed the regio-selective, repeated addition of galactose from lactose to GlcNAc forming the β-galactosyl linkage at the 4-position of the GlcNAc and at the 1-position of D-galactose generating, in addition to GOS, LacNAc, and Galactosyl-LacNAc trisaccharides which were produced by two sequential transgalactosylations. Humanized GOS is chemically distinct from HMOs, and its effects *in vivo* have yet to be determined. Thus, we evaluated its safety and demonstrated the prebiotic's ability to modulate the gut microbiome in 6-week-old C57BL/6J mice. Longitudinal analysis of gut microbiome composition of stool samples collected from mice fed a diet containing hGOS for 5 weeks showed a transient reduction in alpha diversity. Differences in microbiome community composition mostly

within the *Firmicutes* phylum were observed between hGOS and GOS, compared to control-fed animals. In sum, our study demonstrated the biological synthesis of hGOS, and signaled its safety and ability to modulate the gut microbiome *in vivo*, promoting the growth of beneficial microorganisms, including *Bifidobacterium* and *Akkermansia*.

Keywords: galactooligosaccharide (GOS), N-Acetyl-D-lactosamine (LacNAc), safety, human milk oligosaccharides (HMOs), LacNAc synthesis, mouse models

INTRODUCTION

Gut microbial communities play a critical role in the maintenance of host health (1, 2). Hence, beneficial modulation with probiotics (live microorganisms that when administered in adequate amounts provide a benefit to their hosts) (3) and prebiotics (selectively fermented dietary carbohydrate structures that promote the growth of beneficial microorganisms) (4, 5) is desirable and potentially effective translational therapeutics to treat gastrointestinal (GI) diseases linked to disrupted microbial communities (4, 6–9) [reviewed in (10)]. Synbiotics (combinations of prebiotics and probiotics) are also emerging as a focal point of GI biology research, as each component, individually and synergistically, could provide unique benefits reestablishing community resilience and host physiology (11, 12). In previous studies we evaluated highly pure β(1–4) galacto-oligosaccharides (GOS) formulations produced by the optimized version of the hexosyl-transferase gene from *Hamamotoa (Sporobolomyces) singularis* heterologously expressed in *Komagataella* (*Pichia*) *pastoris* (13, 14). This enzyme is one of the most promising catalysts in the field of glycobiology due to its high stability, highly desirable enzymatic properties, and the metabolism of its reaction products (GOS) by specific members of the gut microbial community, impacting its composition and function (15, 16). Beneficial members of the gut microbiota, including *Lactobacillus* and *Bifidobacterium*, hydrolyze GOS via β-galactosidases (17). *Lactobacillus rhamnosus* utilize PTS transporters to internalize GOS prior to hydrolysis (17), while other organisms like specific strains of *Bifidobacterium* (*bifidum*) secrete glycosyl hydrolases to break down complex carbohydrates, internalizing the products of hydrolysis (18). Short-chain fatty acids (SCFAs) generated as the result of GOS assimilation include acetate and lactate (17), which community members, including *Roseburia* and *Faecalibacterium*, can transform into butyrate (6, 19).

LacNAc is an essential component of human milk oligosaccharides (HMOs) and has been demonstrated to be a major bifidogenic factor in the 1950s (20–22). Several HMOs contain lactose (Galβ1-4Glc) at their reducing end, which can be elongated by the addition of β1-3- or β1-6-linked lacto-N-biose (Galβ1-3GlcNAc) or LacNAc (Galβ1-4GlcNAc). Lactose or the oligosaccharide can be then fucosylated by fucosyltransferases in α1-2, α1-3, or α1-4 linkage and/or sialylated by sialyltransferases in α2-3 or α2-6 linkage to yield a variety of terminal structures (23). The study by Yoshida et al. (24) characterized β-galactosidases of *Bifidobacterium longum* subsp. *infantis* to determine how this organism degrades type-1 (lacto-N-biose,) and type-2 (LacNAc,) isomers of HMOs. LacNAc has also been recognized as a building block of glycoproteins and glycolipids in the GI tract. These backbones serve to connect the core structure, which is directly linked to a protein or lipid aglycon with terminal sugars [reviewed in (25)]. LacNAc building blocks and terminal sugars also act as an important precursor of several blood group epitopes (Lewis A, Lewis B, sialyl Lewis A), which are involved in biological processes including fertilization (26), mediation of cell adhesion and pathogen adhesion to colonocytes (27–29).

Chemical and enzymatic synthesis processes have been the most frequently evaluated methods for LacNAc production (30). In recent years, glycoside hydrolases (EC 3.2.1.-) and β-galactosidases (EC 2.1.23) with both hydrolytic and transglycosylation activities, have gained special attention for regio- and stereo-selective synthesis of LacNAc oligosaccharides (https://www.cazypedia.org/index.php/Transglycosylases) (31–37). Enzymatic biosynthesis is considered the most efficient method for producing LacNAc due to specificity, synthesis in one-step reactions, low-cost substrates, sustainability, and overall low environmental impact, [reviewed in (38)]. Conversely, chemical methods to generate LacNAc require multiple reactive hydroxyl groups and laborious protocols for group protection and deprotection to control the stero- and regio-specificities (39, 40). Compared to enzymatic synthesis, generation of LacNAc by chemical synthesis has low yields, a cost-competitive disadvantage for industrial production, hindering the use of LacNAc as an additive in food products (30, 41, 42).

In this study, we describe a novel biological synthesis solution to produce N-Acetyl-lactosamine (LacNAc)-enriched GOS (which we refer to as humanized GOS, hGOS) using optimized *Hamamotoa (Sporobolomyces) singularis* β-hexosyl transferase [rBHT (13, 14)]. The enzyme generates LacNAc-enriched GOS as the product of the reaction between lactose as a galactose donor and N-Acetylglucosamine as acceptor. We first evaluated the efficiency of a *Komagataella* (*Pichia*) *pastoris* cell line carrying membrane-bound β-hexosyl transferase on the generation of GOS and hGOS from lactose and N-Acetyl-glucosamine. Then, conventionally-raised 6-week-old C57BL/6J mice were fed a control diet or modified diets containing GOS or hGOS for 14 days to evaluate its safety and impact on fecal microbial diversity and composition.

MATERIALS AND METHODS

Generation of Dietary Carbohydrate Structures/Prebiotics GOS and hGOS

Membrane-bound β-hexosyl transferase from *Hamamotoa (Sporobolomyces) singularis* in *Komagataella* (*Pichia*) *pastoris* was produced as previously described (13, 14). The standard transgalactosylation reaction utilizing *Komagataella* (*Pichia*) *pastoris* resting cells (harboring membrane-bound enzyme) was initiated by adding standardized amounts of enzyme (1 U g^{-1} lactose) in 5 mM sodium phosphate buffer (pH 5.0) to a similarly buffered solution containing lactose (200 g $liter^{-1}$) and N-Acetylglucosamine (25 g $liter^{-1}$) at 30°C. Reaction products and substrates were analyzed by high-performance liquid chromatography (HPLC) (Shimadzu Corporation, Kyoto, Japan) under isocratic conditions at 65°C and a 0.5-ml min^{-1} flow rate. The mobile phase was water, and separation was performed by two columns in tandem a Supelco gel Ca++ (Supelco, PA), and HPX-42A (Bio-Rad, CA) columns (300 mm by 7.8 mm) coupled to an SPD-20MA and ELSD-LT II detectors (Shimadzu Corporation, Kyoto, Japan). The column was calibrated using galactosyl-lactose (Carbosynth, Berkshire, United Kingdom), LacNAc, Lactose, N-Acetylglucosamine, Glucose, and Galactose (Sigma-Aldrich, St. Louis, MO). Enzymatic activity was determined using 4-nitrophenyl β-D- glucopyranoside or oNP-Glc as substrate as per the previously described methods (13, 14).

Human Equivalent Dose Calculation

The human equivalent dose (HED) for LacNAc was calculated for the animal study using the methods highlighted by the United States Food and Drug Administration and is based on the approximate body weight of the subject (43). The equation used is as follows:

$$HED\left(\frac{mg}{kg}\right) = Animal\ Dose\left(\frac{mg}{kg}\right) \times \frac{Animal\ K_m}{Human\ K_m}$$

Where the K_m factor is a number based on body surface area. For this study, we used an animal dose of 1,500 mg kg^{-1}, based on ∼30 mg LacNAc fed to a ∼20 g mouse per day. Additionally, we used a K_m factor of 3 for mice and 16 to represent a 5 kg human infant (43). Using the formula above, the HED for this study represent an equivalent of 281.25 mg LacNAc per kg body weight in infants. While for a 20 kg child (K_m= 25), the HED would be 180 mg kg^{-1}.

Animal Housing, Treatment, and Sample Collection

All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of North Carolina at Chapel Hill (Approved protocol number: 19-084).

A total of 50 6-week-old C57BL/6J mice were co-housed at random in groups of 5–6 animals and fed a defined diet (D17121301; Research Diets INC.) containing no prebiotics to normalize the gut microbiota for 2 weeks. After a 2-week standardization period, fresh stool samples were collected directly from the anus of each animal into a sterile tube. To avoid cage batch effects, animals were moved into paired housing such that no two animals from the same standardization group were co-housed. Each animal was considered one experimental unit. Upon reassigning housing (**Figure 2A**), animal pairs were split into three distinct groups, each of which began feeding on either the defined control diet (D17121301) ($n = 17$), GOS diet (D17121302) ($n = 17$) in which 71.8 g of cellulose per kilogram diet was replaced with 71.8 g of pure GOS, or hGOS (D18121401) ($n = 16$) where LacNAc represented a 1% (w/w). Composition of each diet is detailed in **Supplementary Table 1**. Each diet was offered *ad libitum* for 2 weeks prior to stool sample collection. Individual animal mass and dietary consumption were measured daily during the first 14 days of the dietary study to assess animal growth and food consumption (total food consumed in a cage/2 = individual animal food consumption) between diet groups. After day-14 sample collection, half of the animals in each treatment group were removed for a tangential study and all remaining animals (total=24, $n = 8$ per diet) continued to consume their respective diets *ad libitum*, with stool sample collections occurring at three additional time points each ∼1-week apart prior to animal sacrifice. At the conclusion of the trial on day 38, each animal was euthanized via CO_2 asphyxiation and cervical dislocation.

Nucleic Acid Isolation

Total DNA was extracted from fecal pellets using the Qiagen ClearMag Extraction system on KingFisher Flex Magnetic Bead processing instrument as described (15). Briefly, stool samples were transferred to a screwcap tube containing 10 mg of sterile acid-washed glass beads (0.1–0.5 mm diameter) and 700 µl PM1 solution (Qiagen, Valencia, CA). Samples were homogenized for 5-min at 15 Hz in Qiagen Tissue Lyser II (Qiagen). Bead-beaten samples were treated with IRS-PCR inhibitor remover solution (Qiagen) (3:1; lysate:IRS ratio) overnight at 4°C and transferred to KingFisher Deep-well plate containing ClearMag magnetic beads and binding buffer (Qiagen). Sample plates were subsequently processed on KingFisher Flex instrument to isolate and wash DNA. DNA was quantified with Quant-iT™ PicoGreen® dsDNA reagent (Molecular Probes, Thermo Fisher Scientific, Waltham, MA) and stored at −20°C.

16S rRNA Amplicon Sequencing

Total DNA was subject to amplification of the V4 variable region of the 16S rRNA gene using primers 515F and 806R (44) with Illumina adaptors. Amplicons were barcoded using Illumina dual-index barcodes [Index 1(i7) and Index 2(i5)], purified using Agencourt® AMPure® XP reagent (Beckman Coulter, Brea, CA) and quantified with Quant-iT™ PicoGreen™ dsDNA Reagent (Molecular Probes, Thermo Fisher Scientific). Libraries were pooled in equimolar amounts and sequenced on HiSeq2500 instrument (Illumina, San Diego, CA).

Sequencing Data Analysis

Analysis of 16S rRNA amplicon sequencing data was carried out using the QIIME2 pipeline as described (45). Briefly, sequences were grouped into Operational Taxonomic Units (OTUs) using UCLUST (46). OTU sequences were aligned, and phylogenetic

trees were built (47). Before generating the phylogenetic tree, the overall OTU table was collapsed using the "taxa collapse" plugin in QIIME2. The set of representative sequences was then trimmed to include only one representative sequence for each collapsed OTU. The filtered set of representative sequences was then aligned using MAFFT, and a phylogenetic tree was generated from the alignment using RAxML (48, 49). The phylogenetic tree was finally annotated with presence/absence data using iToL and PhyloToAST (50–52). Alpha and beta diversity metrics were calculated in R 4.0.3 using the phyloseq and vegan packages (53–55). Only data from young animals (6 weeks old) were used in the calculation of diversity metrics. Both the Shannon entropy and inverse Simpson indexes were calculated to ensure an accurate estimation of the true alpha diversity of the samples. Beta diversity was calculated using principal coordinate analysis (PCoA) of the weighted UniFrac distances (56).

Statistical Analysis

Data were evaluated for homogeneity of variance using Levene's test. Statistical significance of alpha diversity was evaluated using a repeated-measures ANOVA followed by Tukey's Honest Significant Difference test to separate means. The 95% confidence ellipses for beta diversity plots were calculated in R 4.0.3 using ggplot2 (57). Beta diversity statistical analyses were performed using the PERMANOVA and PERMDISP functions of the vegan package in R 4.0.3. All statistical analysis results for the alpha and beta diversity analyses can be found in **Supplementary Table 2**. The α for all statistical tests was fixed at 0.05.

Availability of Data and Materials

All sequencing data has been submitted to the NCBI repository and can be accessed via the following accession number: PRJNA681811.

RESULTS

rBHT Catalyzed the Repeated Addition of Galactose From Lactose to N-Acetylglucosamine

The reactions catalyzed by the rBHT enzyme were regio-selective, forming the β-galactosyl linkage at the 4-position of the GlcNAc and the 1-position of D-galactose, synthesizing various glycoconjugates directly from soluble GlcNAc. The obtained products, in addition to GOS, included Gal-β(1, 4)GlcNAc (LacNAc, **Figure 1A**, panel B) disaccharides and Galβ-(1, 4)Galβ-(1, 4)GlcNAc (Galactosyl-LacNAc, **Figure 1A**, panel C) trisaccharides, which were produced by two sequential transgalactosylations. **Figure 1B** shows the kinetics of the reaction performed during 8 days of incubation using rBHT polypeptides (e.g., whole cells displaying membrane-bound enzyme). The enrichment of GOS with LacNAc at a ratio lactose/N-Acetylglucosamine of 8:1 performed for these experiments (200 g/L lactose and 25 g/L GlcNAc) generated 25 g/L of LacNAc and 100 g/L hGOS after 48 h of incubation. At this time point, the reaction was terminated, and the products of the reaction (hGOS) were freeze-dried.

Animal Health and Diet Consumption

We conducted an animal experiment with conventional 6-week-old C57BL/6J mice fed a control diet, or modified diets containing GOS or hGOS to assess their impact on the gut microbiome (**Figure 2A**). Results showed no impact of GOS or hGOS diets on weight or daily food consumption. Each mouse consumed ∼3 g of food per day, with no significant differences between the diets (**Supplementary Figure 1**). Therefore, based on the formulation of each prebiotic diet, we calculated that the dose of prebiotic consumed per day by each mouse was 0 mg/day (on control diet), and ∼190 mg/day of total prebiotic (GOS, hGOS). This estimate translates to ∼171 mg/day of GOS (GOS diet) and ∼30 mg/day of LacNAc (hGOS diet) based on prebiotic formulation data.

Modulation of the Gut Microbiota by hGOS

After 2 weeks of feeding on diets containing prebiotics, animals exhibited a significant (repeated measures ANOVA $p < 0.05$) reduction in alpha diversity (**Figure 2B**). Over the length of the study, diversity of prebiotic-fed animals returned to values comparable to the control diet with no statistically significant differences between groups at day 28. PCoA plots revealed distinct clustering of hGOS-fed animals, which displayed a much tighter dispersion pattern compared to control animals (PERMDISP $p = 0.009$), suggesting a higher similarity between communities within hGOS-fed than control-fed animals (**Figure 2C**). Spatial medians were significantly different between groups (PERMANOVA $F = 7.6463$, $p \leq 0.001$).

Taxonomy plots of relative microbial abundance revealed the genus-level variability between animals fed control diets, and those consuming either GOS or hGOS diets over time (**Figure 3A**). The most dramatic changes in the assembled microbial communities were observed between timepoint 0 and 14 days after introducing prebiotic diets. Changes between the communities within prebiotic-fed animals after 14 days were minimal. Analysis of Composition of Microbiome (ANCOM) used to further explore microbial abundance changes within the communities of prebiotic-fed animals across all time points revealed an increased relative abundance of beneficial microorganisms including *Akkermansia*, *Bifidobacterium*, and *Bacteroides*, along with *Allobaculum* in both GOS and hGOS diets. The dietary interventions reduced the relative abundance of *Butyricicoccus*, *Clostridium*, *Turicibacter*, and *Lachnospiraceae* across all time points (**Figures 3A,B**, **Supplementary Figure 2**).

Figure 4 shows a phylogenetic tree generated using PhyloToAST that includes 117 unique OTUs detected in at least one of the three diets examined. Of the 117 unique OTUs detected, 76 OTUs were detected in all conditions (Control, GOS, hGOS), and 40 were detected in control and GOS but not in hGOS fed mice, while none was detected in control and hGOS but not GOS fed mice. The majority of the OTUs detected in the control and GOS mice, but not in hGOS mice, belonged to the *Firmicutes* phylum. Taxa not detected in the hGOS group compared to control and GOS group within the Phylum *Firmicutes* and Class *Bacilli* included *Lactobacillus reuteri, L. zeae*, species of *Enterococcus, Brevibacillus,*

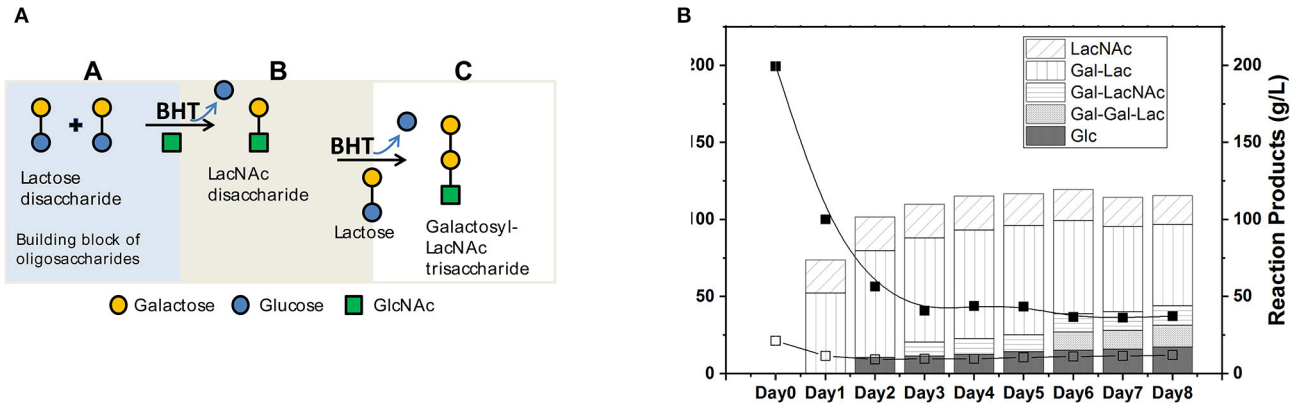

FIGURE 1 | (A) Illustrative representation of catalysis of hGOS from carbohydrate constituents. **(B)** Time course evolution in g/L of dietary carbohydrate structures generation and residual substrates in g/L over 8 days; Lactose (Lac), N-Acetylglucosamine (GlcNAc), and Glucose (Glc). The products of the reaction were N-Acetyl-lactosamine (LacNAc), Galactosyl-β(1–4)lactose (Gal-Lac), Galactosyl-β(1–4)N-Acetyl-lactosamine (Gal-LacNAc), and Galactosyl-β(1–4)Galactosyl-β(1–4)lactose (Gal-Gal-Lac). The reactions were performed using whole cells membrane-bound protein (1 U rBHT.g^{-1} lactose). The initial conditions of the reactions contained 200 g/L lactose; 25 g/L N-Acetylglucosamine (GlcNAc), in 5 mM sodium phosphate buffer (pH 5.0) and incubated at 30°C. Samples were removed periodically and separated by HPLC and quantified with sequential ELSD and PDA detectors.

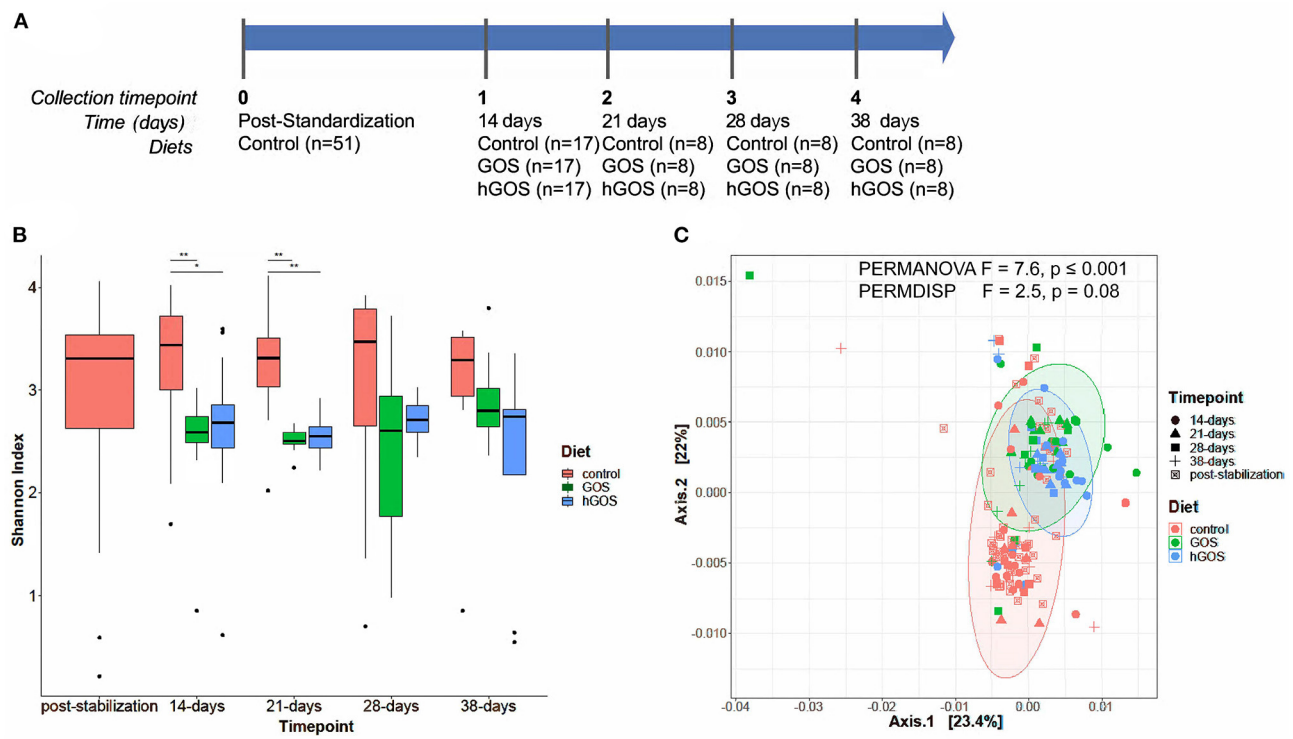

FIGURE 2 | (A) Experimental timeline of the animal study, delineating the number of animals in each experimental group, and the duration between sample collection and analysis. **(B)** Box plots show changes in Shannon diversity values between groups fed control (red), GOS (green), or hGOS (blue) diets over time. Significant differences in Shannon diversity between diets are indicated by bars and asterisks, with *indicating $p < 0.05$ and **indicating $p < 0.01$. Only statistically significant differences are shown. **(C)** PCoA, PERMANOVA, and PERMDISP analyses of samples between time points show significant differences in clustering as a function of the diet.

Paenibacillus, Anaerobacillus, Virgibacillus, Facklamia, Unclassified Lactobacillales, Bacillales, Enterococcaceae, and *Planococcaceae.* Within the Phylum *Firmicutes* and Class *Clostridia*, the following were not detected in the hGOS group: *Veillonella dispar, Ruminococcus flavefaciens, Faecalibacterium prausnitzi,* species of *Butyrivibrio, Pseudobutyrivibrio, Lachnospira, Oxobacter, Roseburia, Dialister, Veillonella, Phascolarctobacterium, Anaerotruncus, Blautia* and Unclassified *Clostridiales, Clostridiaceae,* and *Veillonellaceae.* Only 3 OTUs corresponding to the Phylum

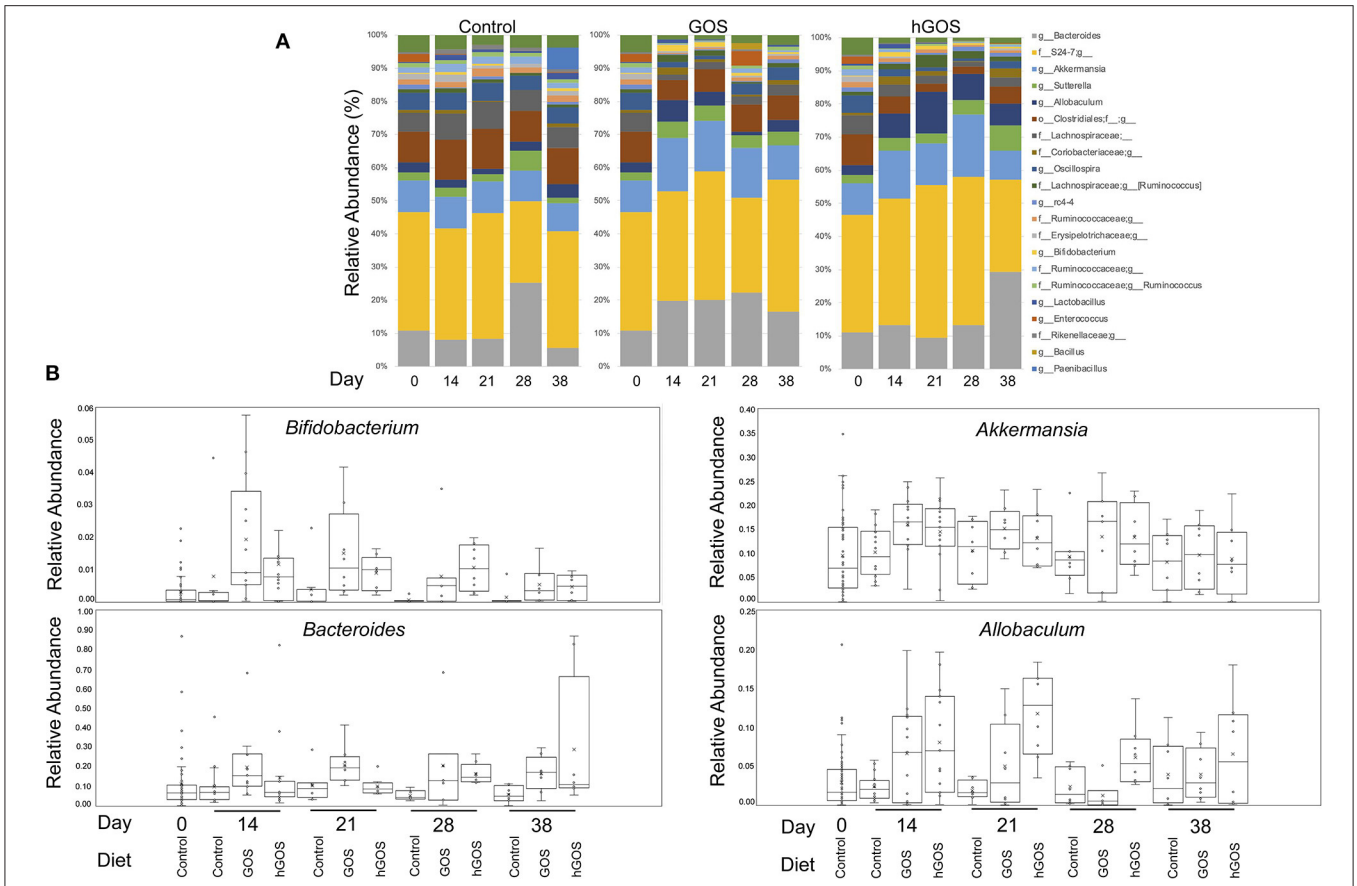

FIGURE 3 | (A) Genus-level taxonomy plots reveal highly abundant taxa in each group at each time point in the study. Changes associated with diet were observed immediately (day 14) and were persistent throughout the trial. **(B)** *Bifidobacterium*, *Akkermansia*, *Bacteroides*, and *Allobaculum* were significantly increased in prebiotic-fed animals at 14 days compared to controls.

Bacteroidetes (*Bacteroides eggerthii, B. caccae, and B. fragilis*) and 7 Proteobacteria (*Burkholderia bryophila, Acinetobacter, Bilophila, Hydrogenophaga, Pseudomonas, Halomonas*, and Unclassified *Enterobacteriaceae*) were not detected in the hGOS group. **Supplementary Table 1** presents the mean relative abundance values for each observed OTU in each of the experimental conditions.

DISCUSSION

Prebiotics, including GOS, are selectively fermented by gut microorganisms and promote the growth of beneficial microorganisms when consumed in adequate amounts (16, 17). In this study, we report the biological synthesis of hGOS enriched in LacNAc and determined its lack of adverse effects by determining the impact of feeding on the gut microbiome of healthy 6-week-old C57BL/6J mice in comparison with defined control and GOS-containing diets.

Traditionally, higher values of gut microbiota diversity has been associated with good health (58–60). In our study, feeding of both GOS and hGOS-enriched diets initially reduced diversity, even when constituents of the gut microbial community considered beneficial (*Bifidobacterium*, *Akkermansia*, and species of *Bacteroides*) increased. Diversity increased at 28 and 38 days, suggesting that sustained hGOS feeding would lead to a diversity comparable to the control group. In addition, we did not observe differences in dietary consumption or weight in prebiotic-fed animals compared to the control group. We have recently reported an initial decreased diversity in 6- and 60-week old GOS-fed C57BL/6J mice after 2 weeks (15), which is in accordance with studies of GOS-supplemented infant formula (61) but contrast with other studies on human adults (6, 62) and young or adult BALB/c mice that showed no changes on diversity due to GOS feeding (63, 64). Considering the biochemical structure of GOS and hGOS and their similarity to HMOs, it makes sense that these prebiotics exert a restrictive selection of microorganisms to only microbes capable of establishing a mutualistic relationship with the host as observed in breastfed infants (65). The restrictive colonization effect leads in babies to the successive establishment of different bacterial groups, from aerotolerant bacteria to progressively stricter anaerobes (66, 67), and could provide in adults and older adults a strategy to beneficially modulate the gut microbiome by the subsequent introduction of microbial network units (68).

Members of the gut microbial community including strains of *Bifidobacterium* and *Lactobacillus* encode galactosidases

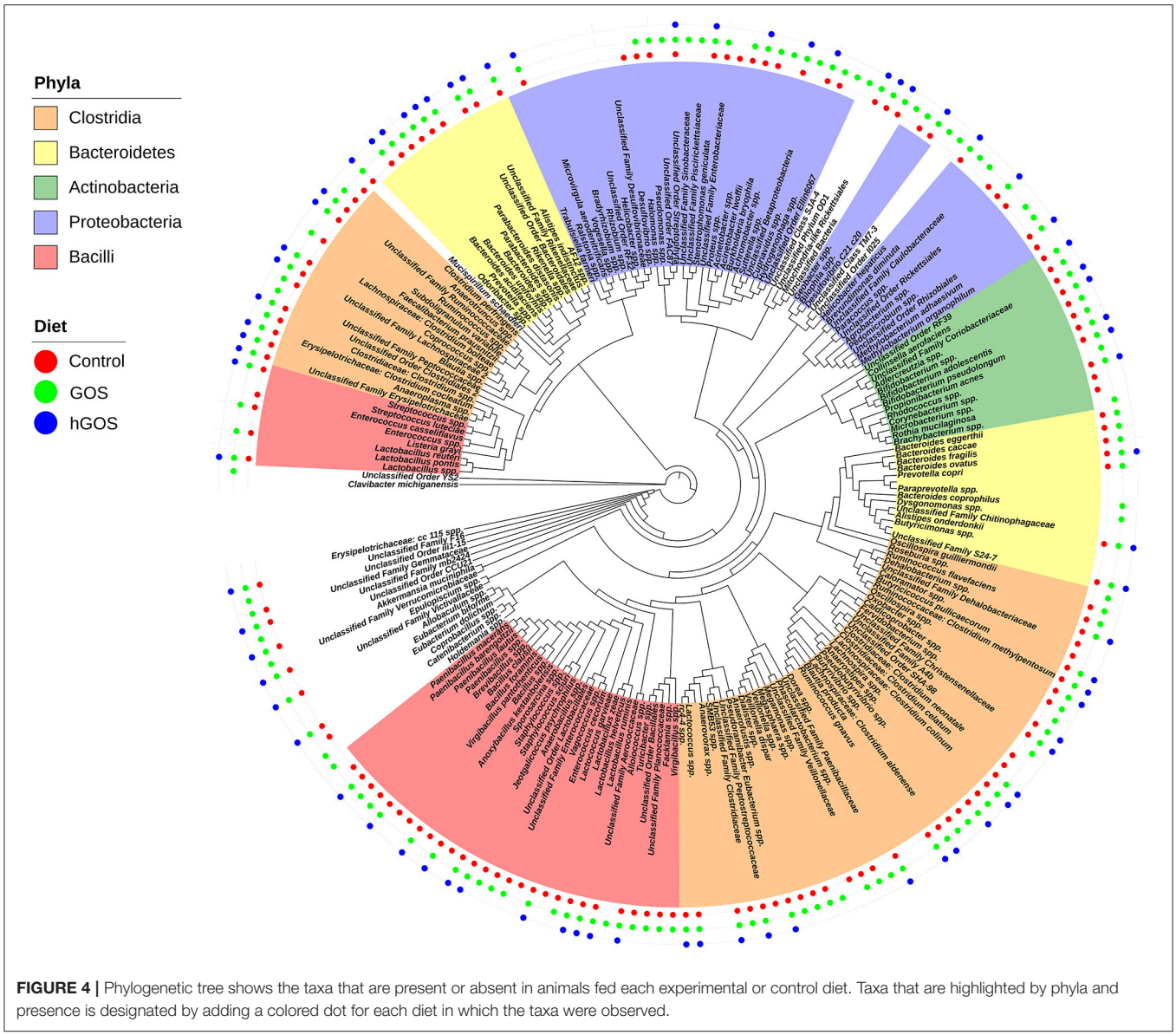

FIGURE 4 | Phylogenetic tree shows the taxa that are present or absent in animals fed each experimental or control diet. Taxa that are highlighted by phyla and presence is designated by adding a colored dot for each diet in which the taxa were observed.

genes that hydrolyze complex carbohydrates including GOS, as demonstrated in our previous and current studies (15–17, 19, 69) generating products which other members of the gut microbiota can further utilize through cross-feeding (19, 70). Due to structural similarities between the dietary carbohydrate structures contained in GOS and hGOS, it can be expected that their hydrolysis will result in similar molecules, including lactate and acetate, which could subsequently be utilized to generate other SCFAs of biological relevance, including butyrate. We anticipated that the additional LacNAc residues in hGOS would provide an additional substrate for bacterial enzymatic systems, allowing for different microorganisms to utilize these compounds compared to GOS. However, our study was not able to detect bacterial groups that used hGOS but not GOS. Further experiments will be required to characterize the gut bacterial metabolism of hGOS. Among other changes in the gut microbiota, feeding GOS and hGOS increased the abundance of *Akkermansia muciniphila*, a microorganism that predominantly utilizes mucin as its energy source. GOS enrichment of *Akkermansia* is likely a consequence of increased mucin production (15). However, hGOS (containing LacNAc) may be utilized directly by *Akkermansia* due to a similar LacNAc structure found in hGOS and mucin (71). These findings are consistent with our previous animal studies (15, 16); however, animal models have significant limitations due to fundamental differences between human and mouse-originated microorganisms (72). Further studies are currently underway to better assess the impacts of hGOS on human bacterial isolates, with the ultimate goal of developing a prebiotic optimized for human consumption.

Finding the proper dose of a new therapeutic compound is vital not only to ensure safety and efficacy in clinical trials but

is also necessary to ensure the economic feasibility of the new product. For GOS, a low dose (below 2 g per day) may not elicit the desired modulatory effect (6, 73), while an excessively high dose (over 15 grams per day) may induce undesired GI effects (6, 74). Studies have shown the importance of translating the dose of a compound validated in animal models to the HED [reviewed in (75)]. Here, we demonstrated the lack of adverse effects of a HMO mimetic composition, LacNAc-enriched GOS, and its ability to modulate the gut microbiome at a HED of 180 mg kg^{-1} day^{-1}. The values tested during our experiments are in accordance with the recommended values by the EFSA panel (76) for 2′-O-fucosyllactose (2′-FL) and lacto-N-neotetraose (LNnT). The tested HED was six times higher than the calculated average amount of LacNAc consumed in a day by a 5 kg infant (1,400 vs. 232 mg day^{-1}), potentially highlighting the lack of adverse effects of LacNAc, even at a higher-than-physiological doses (77, 78).

Breast milk is undoubtedly the optimal source of nutrition for the human infant (79) and, until recently, the HMOs present in mother's milk could not be replicated in enough quantities to add to infant formulas. Five years ago, the study by Marriage et al. (80) showed that weight, length, head circumference growth and uptake of 2′FL, measured in the blood and urine, were similar to those of breastfed babies and today, some infant formulas have already incorporated this HMO. As a major building block of HMOs, the addition of LacNAc to the existing list of prebiotic compounds is of paramount importance for the further development of safe, nutritionally, and immunologically complete formulas. Hence, our study represents the first step in evaluating the safety and efficacy of enzymatically produced hGOS in an animal model of weaned human infants.

DATA AVAILABILITY STATEMENT

All sequencing data has been submitted to NCBI repository and can be accessed via the following accession number: PRJNA681811.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill (Approved protocol number: 19-084).

AUTHOR CONTRIBUTIONS

JA performed and analyzed animal experiments. HW and JR curated and analyzed the amplicon sequencing data presented in the manuscript. SD produced the humanized GOS for the animal experiments. MA-P and JB-B designed the experiments and edited the manuscript. All authors contributed to the writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.640100/full#supplementary-material

Supplementary Figure 1 | Bodyweight and daily dietary consumption datasets are included to show that animal growth and intake were not inhibited by the introduction of either experimental diet (GOS and hGOS).

Supplementary Figure 2 | Bacterial taxa shown to have a significantly reduced relative abundance in the presence of hGOS.

Supplementary Table 1 | Composition of diets used in the animal study.

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REVIEW
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Potential Applications of Endo-β-*N*-Acetylglucosaminidases From *Bifidobacterium longum* Subspecies *infantis* in Designing Value-Added, Next-Generation Infant Formulas

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Human milk is the optimal source of infant nutrition. Among many other health benefits, human milk can stimulate the development of a *Bifidobacterium*-rich microbiome through human milk oligosaccharides (HMOs). In recent years, the development of novel formulas has placed particular focus on incorporating some of the beneficial functional properties of human milk. These include adding specific glycans aimed to selectively stimulate the growth of *Bifidobacterium*. However, the bifidogenicity of human milk remains unparalleled. Dietary *N*-glycans are carbohydrate structures conjugated to a wide variety of glycoproteins. These glycans have a remarkable structural similarity to HMOs and, when released, show a strong bifidogenic effect. This review discusses the biocatalytic potential of the endo-β-*N*-acetylglucosaminidase enzyme (EndoBI-1) from *Bifidobacterium longum* subspecies *infantis (B. infantis)*, in releasing *N*-glycans inherently present in infant formula as means to increase the bifidogenicity of infant formula. Finally, the potential implications for protein deglycosylation with EndoBI-1 in the development of value added, next-generation formulas are discussed from a technical perspective.

Keywords: human milk oligosaccharides, *N*-glycans, endo-β-*N*-acetylglucosaminidase, bifidobacteria, infant formula

INTRODUCTION

Human milk is the optimal source of infant nutrition. It provides all the energy, nutrients, and bioactive compounds required for the growth and development of the infant. Human milk feeding is associated with numerous benefits, including a reduced risk of gastrointestinal and respiratory infections and improved immune development (1). Given the known benefits of human milk, there is a great interest in improving infant formulas to resemble the compositional profile of human milk (2) and reduce the relative deficits associated with infant formula consumption. Thus, a better understanding of human milk components and their biological functions is paramount to the improvement of infant formulas (3, 4).

One of the most significant differences between human milk-fed and formula-fed infants is the composition of the gut microbiome (4–6). Breastfed infants have a less diverse yet more stable microbiome, and certain species of infant-adapted bifidobacteria can reach up to 90% of total fecal microbiome (7–9). On the other hand, the microbiome of the formula-fed infants is more variable (8, 10). To mitigate these differences between infant formula and human milk, most formulations add prebiotics such as galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) (11, 12) and/or probiotics. Probiotics added to formula are currently limited to *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* (13). Human milk contains complex carbohydrates known as human milk oligosaccharides (HMOs). HMOs are not digested in the small intestine and reach the colon intact where they are fermented by specialized species of bifidobacteria (14). However, most prebiotic compounds added to formula are not selective for the growth of bifidobacterial (15). Thus, the difference in oligosaccharide content in human milk and infant formula is likely to explain, at least in part, the compositional differences in the microbiome of formula-fed and human milk-fed infants.

Recently, synthetic HMOs such as 2′-fucosyllactose (2′FL) and lacto-*N*-neotetraose (LNnT) have been added to infant formula with the intent to increase the bifidogenic effect of infant formula (16–19). However, HMO fortification of infant formulas has remained low when compared to the global average concentration of HMOs in human milk. On the other hand, little attention has thus far been given to *N*-glycans, which are naturally found as glycoconjugates in both human and bovine milk proteins and bear striking structural and compositional similarity to HMOs. Owing to both their compositional and structural similarities to HMOs, *N*-glycans derived from milk glycoproteins have been shown to be selectively bifidogenic. In this review, we describe human milk as a complex biofluid. We then describe the types, compositions, and indications for most infant formulas available in the market. Finally, we propose the use of specialized enzymes known to be active in the gut microbiome of breastfed infants colonized with *Bifidobacterium* in order to improve the bioavailability of *N*-glycans in infant formula and we discuss potential applications for the design on next-generation infant formulas to improve the suitability of infant formulas for *Bifidobacterium*.

TABLE 1 | Human milk composition.

Component	Amount	References
Human milk composition		
Energy	65–70 kcal/dL	(3)
Lactose	67–78 g/L	(3)
Protein	9–12 g/L	(3)
Lipid	32–36 g/L	(3)
Vitamins		
Vitamin D	4–40 IU/L	(23)
Vitamin C	30.3 ± 6.7 mg/L	(24)
Vitamin K	0.9–6.9 mg/L	(23)
Minerals		
Calcium	84–462 mg/L	(25)
Magnesium	15–64 mg/L	(26)
Phosphorus	17–278 mg/L	(25)
Sodium	512 mg/L	(23)
HMOs	5–15 g/L	(21, 22, 27)
Lactose		
2′-Fucosyllactose (2′FL)		
3′-Fucosyllactose (3′SL)		
6′-Siayllactose (6′SL)		
3′-Sialyllactose (3′SL)		
Lacto-*N*-tetraose (LNT)		
Lacto-*N*-neotetraose (LNnT)		
Lacto-N-hexaose (LNH)		
Lacto-*N*-fucopentaose I (LNFP I)		
Lacto-*N*-fucopentaose II (LNFP II)		
Lacto-*N*-fucopentaose III (LNFP III)		
Lacto-*N*-fucopentaose V (LNFP V)		
Siayllactose-*N*-tetraose b (LST b)		
Siayllactose-*N*-tetraose c (LST c)		
Disiayllacto-*N*-tetraose (DSLNT)		
Fucosyllacto-*N*-hexaose (FLNH)		
Difucosyllacto-*N*-hexaose (DFS-LNH)		

MACRONUTRIENTS IN HUMAN MILK

The composition of human milk is dynamic, and it has evolved to provide optimal infant nutrition. Human milk contains macronutrients including proteins, lipids, carbohydrates, and micronutrients such as vitamins and minerals. It also contains non-nutritional bioactive components, growth factors, hormones, immunological factors, noncoding RNAs, and microorganisms (20). The macronutrient composition of human milk ranges from 9 to 12 g/L protein, 32 to 36 g/L lipids, 67 to 78 g/L lactose, and 5 to 15 g/L HMOs (3, 21, 22) (**Table 1**).

Proteins in human milk comprise two major classes, caseins, and whey (28). The main casein proteins are α-, β-, and κ-casein, and whey proteins are α-lactalbumin, lactoferrin, immunoglobulins (Igs), serum albumin, and lysozyme (29, 30). Non-protein nitrogen-containing compounds including urea, uric acid, creatine, creatinine, amino acids, and nucleotides represent ∼25% of human milk nitrogen (31).

Fat is the largest source of energy in human milk, contributing to 40–55% of the total energy provided by human milk. Triacylglycerols contribute ∼98% of human milk fat. More than 200 fatty acids are present in human milk with different concentrations (32). Palmitic and oleic acids are the most abundant fat types in human milk (33). The content of fatty acids, particularly the long-chain polyunsaturated fatty acids (LCPUFAs), is mostly affected by maternal diet.

Lactose is the main nutritional carbohydrate in human milk comprising 67–78 g/L and supplies approximately half of the energy obtained in by the infant. The other significant carbohydrate fractions of human milk are HMOs. However, contrary to that of lactose, the concentration of HMOs varies

depending on the stage of lactation and maternal genetic factors, ranging from 5 to 15 g/L (34).

HUMAN MILK OLIGOSACCHARIDES (HMOs)

HMOs are non-nutritive, functional, and complex carbohydrates in human milk. The composition of HMOs in human milk is influenced by maternal genetic and lactation stage (35). Nearly 200 distinct oligosaccharides have been described to date (36). The basic core structure of HMOs includes disaccharide lactose at the reducing end, which is elongated with *N*-acetyllactosamine units, by the action of specific glycosyltransferases in the mammary gland. HMOs are composed of both neutral and anionic species with five monosaccharides as building blocks. These building blocks are D-glucose (Glc), D-galactose (Gal), *N*-acetylglucosamine (GlcNAc), L-fucose (Fuc), and *N*-acetylneuraminic (or sialic acid; NeuAc). The length of the HMO chains varies from three to fifteen carbohydrate units, and HMO structures can be either linear or branched forms (37, 38). There are three main HMO categories: neutral *N*-containing (non-fucosylated) (42–55%), neutral (fucosylated) (35–50%), and acidic (sialylated) (12–14%) (39).

2′-3-Fucosyllactose (FL) or 3′-6′-sialyllactose (SL) is formed when the lactose core is conjugated with Fuc or NeuAc. The lactose core is coupled to repeats of lacto-*N*-biose (Galβ1-3GlcNAc; LNB), and these chains are known as type 1 chains. The most abundant HMO is lacto-*N*-tetraose (LNT) as type 1 (40). When an *N*-acetyllactosamine unit (LacNAc; Galβ1-4GlcNAc) is conjugated to the lactose core, the type 2 chain is formed. Lacto-*N*-neotetraose (LNnT) is a type 2 chain in HMOs. Type 1 chains in HMOs are more abundant than those of type 2. Type 1 and 2 chain HMOs could be further elongated with fucosyl and sialyl residues in α-linkages to form hexoses, octaoses, and larger HMOs and together represent ∼70% of all human milk oligosaccharides (34, 41) (**Table 1**). These alterations increase the number and complexity of HMO structures (38, 42).

Functions of HMOs

HMOs are hypothesized to have many important roles in infant innate defense, metabolic health, and neural development (43–45). Clinical and *in vitro* studies suggest that HMOs may block pathogen adhesion by serving soluble ligand analogs (43, 46, 47). As HMOs have structural features that mimic epithelial surface carbohydrates, they are thought to also serve as decoy receptors for pathogens (46, 48–50). HMOs are also thought to promote several intracellular processes like differentiation and apoptosis of intestinal epithelial cells (51). They can also have direct bactericidal or bacteriostatic effects. For instance, some HMOs can directly inhibit the *in vitro* growth of *Streptococcus agalactiae*, a known invasive bacterial pathogen in newborns (27, 52); other HMOs have been demonstrated to reduce pathogen adherence to colonic cells *in vitro* (53). Specific components present in HMOs (e.g., sialic acid) are also critical for the development of neurons and brain development, as well as neuronal transmission, cognitive ability and synaptogenesis (45, 54, 55).

One of the most well-characterized functions of HMOs is to serve as a prebiotic source and shape the microbial community of the infant gastrointestinal tract (56). HMOs reach the colon undigested where they are utilized by specialized gut microbes (57) that possess the necessary molecular machinery for transport and metabolization of these complex structures. Specific species of infant-adapted bifidobacteria [*Bifidobacterium longum* subsp. *infantis* (*B. infantis*), *Bifidobacterium bifidum* (*B. bifidum*), *Bifidobacterium breve* (*B. breve*), and *Bifidobacterium longum* subsp. *longum* (*B. longum*)] have the capability to degrade and utilize oligosaccharides and thus often become the most dominant species in the breastfed infant gut (58–61). Short-chain fatty acids (SCFA) (acetate, propionate, and butyrate) are produced as a result of fermentation of HMO in the colon. These molecules create an acidic environment (low pH) which favors the growth of strains of bifidobacteria while concomitantly creating an unfavorable environment for the growth of pH-sensitive pathogens (7, 41, 62).

HUMAN MILK GLYCOPROTEINS AND THEIR FUNCTIONS

Glycosylation is a diverse and common type of posttranslational modification that involves the attachment of a saccharide chain to a protein structure (63, 64). Approximately 70% of human milk proteins are found in glycosylated forms including lactoferrin, lysozyme, bile salt-stimulated lipase (BSSL), secretory IgA (SIgA), casein, and α-lactalbumin (65, 66). Several preclinical and clinical studies suggest that human milk glycoproteins have key roles in infant development. For instance, osteopontin is involved in regulating mineral deposition and osteoclasts activity in the bones (67); insulin-like growth factors participate in the processes related to the development of the intestinal mucosa (68); bile salt-stimulated lipase aids milk fat digestion (69); lactoferrin facilitates iron uptake in the small intestine (70); and β-casein-based phosphopeptides facilitate calcium absorption (71, 72).

Human milk glycoproteins may also have roles in protecting infants against pathogen infection (73–75). Lactoferrin has been reported to have bacteriostatic and bactericidal effects (76, 77). Lysozyme cleaves glycosidic linkage in the peptidoglycan structure of bacterial cell walls, providing innate protection against microbial infections (78). Interestingly, the level of lysozyme susceptibility varies between different bifidobacteria strains (79, 80). Some bifidobacteria strains of human infant origin are more resistant to lysozyme relative to animal and dairy-derived strains (81). This may suggest that lysozyme in human milk acts as a selection factor for coevolved bifidobacteria in the infant gut, such as *B. infantis* (80, 82, 83). Another predominant human milk protein is SIgA. SIgA acts as a protective defense against pathogens in the infant gut (74, 84). Other human milk glycoproteins, including BSSL and lactadherin, also have protective effects on the infant's health (74). Notably, BSSL has been associated with inhibition of Norwalk virus, a common cause of gastroenteritis, *in vitro* (85).

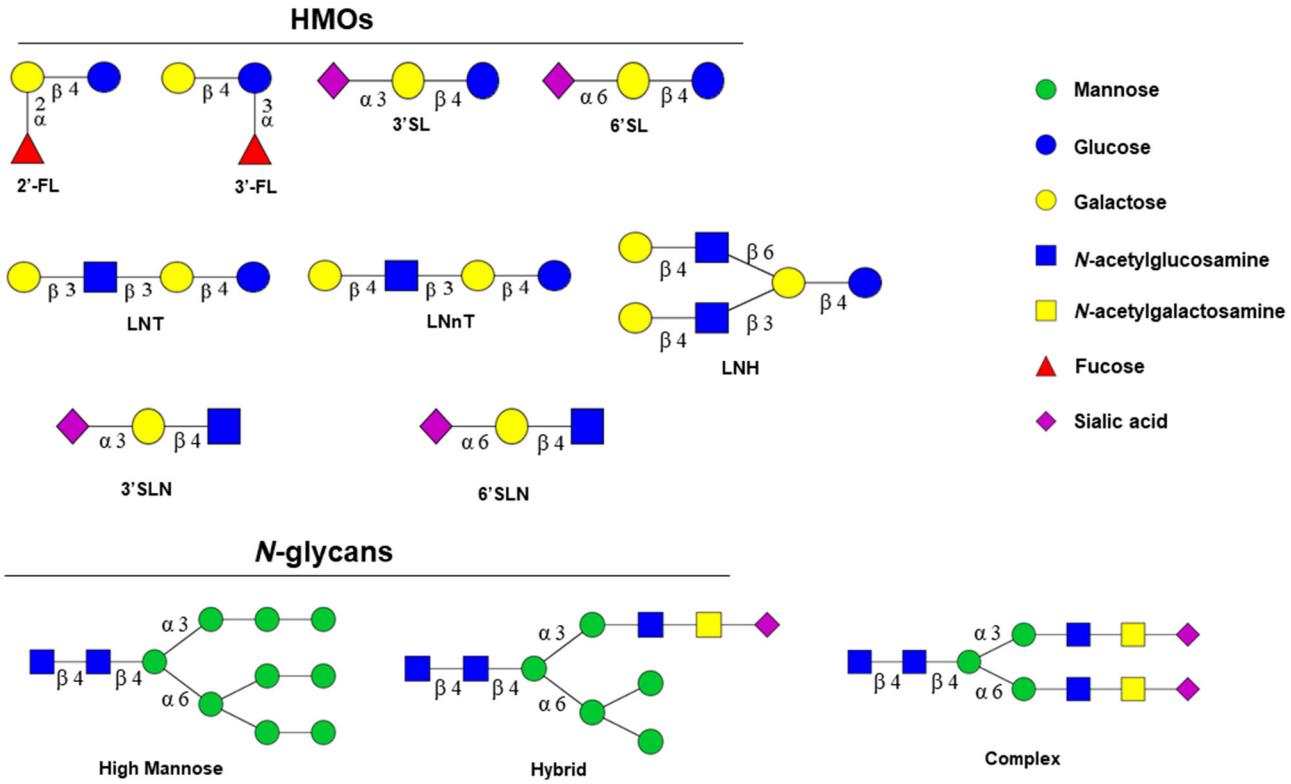

FIGURE 1 | Structural similarities between HMOs and *N*-glycans. Structurally, *N*-glycans are bound to the amide group of asparagine (Asn) residue of the proteins via *N*-acetylglucosamine (HexNAc) in a specific amino acid sequence Asn-X-Ser/Thr or Asn-X-Cys (cysteine) (where X could be any amino acid except proline) (87, 88). *N*-glycans consist of a single core that has two *N*-acetylglucosamine (GlcNAc) followed by three mannoses. Further glycosylation determines the types of *N*-glycans that are classified into three main classes: high mannose (HM), hybrid (HY), and complex type (CT) based on composition (89); HM glycans typically contain unsubstituted terminal mannose sugars and 5–9 mannose residues attached to the chitobiose ($GlcNAc_2$) core.

The glycan structures found on these glycoproteins are strikingly similar to HMOs, in both their monosaccharide composition and linkage types (86) (**Figure 1**). *N*-glycans also form complex structures which increase their specificity. This may explain why *N*-glycans isolated from human and bovine milk are bifidogenic (90), although not equally across bifidobacterial species (91). Specifically, *N*-glycans released from bovine milk glycoproteins selectively stimulates the growth of infant-adapted *B. infantis* whereas *B. animalis*, associated with an animal origin, is not capable of utilizing these structures (91). Further, a recent *in vivo* study showed that 19 unique *N*-glycan structures that are attached to lactoferrin and immunoglobulins stimulate the growth of *B. infantis* (92). Similar to HMOs, *N*-glycans are fermented into SCFAs, mainly lactate, acetate, and also butyrate and propionate (93). The colonic epithelium and microbial ecosystem can be affected from these end products by absorbing SCFAs and lowering the pH of the ecosystem (93). These metabolites primarily lactate and acetate lower the intestinal pH providing resistance to microbial colonization (7, 62, 94). Importantly, fermentation of *N*-glycans into acidic end-products, such as acetate and lactate, disfavors the growth of bacteria that degrade gastrointestinal mucin, and contributes to a considerable reduction in potentially pathogenic bacteria (7, 94–96). This is because most pathogenic bacteria preferentially grow near neutral pH (pH: 6.0–7.0) or grow under acidic conditions inefficiently (97). Therefore, the establishment of the gut microbiome by limiting pathogenic bacterial composition maximizes nutrition for other microbes and reduces inflammation, virulence factors, and antibiotic-resistant genomes (ARGs) in the gut environment. Thanks to the results of the fermentation and these metabolites, colonization of probiotic bacterial level in the gut microbiome, especially *Bifidobacterium* and genes conferring utilization of *N*-glycans, significantly increases. Thus, the development of the gut microbiome by providing colonization resistance to intestinal pathogens is critical for the development of the infant gut microbiome (94, 98).

INFANT FORMULA AND *N*-GLYCANS

Infant formulas are intended as an effective breast milk substitute and are formulated to mimic nutritional composition, including macro- and micronutrients as well as bioactive components, of human milk (99). Most infant formulas are manufactured from bovine milk. The nutritional composition of all infant formulas must follow the global standards as recommended by the European Society for Pediatric Gastroenterology, Hepatology,

TABLE 2 | Types of infant formulas and their properties.

Type of formula	Key product features	Intended
Routine use	Conventional protein, fat, and carbohydrate composition to support healthy growth and development, meeting the requirements, for example, of the US Infant Formula Act, 1980 (104)	Suitable for most term infants, when breast milk is not an option
Premature	May contain partially hydrolyzed whey and carbohydrate source lactose.	Premature and low birth weight infants, where donor milk or mother's own milk is not available
	Higher calcium, phosphorus	
Allergy management	1. Extensively hydrolyzed casein and/or whey	Infants with allergy based on cow milk protein, where breast milk is not an option
	2. 100% free amino acids. No peptides	Infants with bovine milk protein hypersensitivity even with extensively hydrolyzed cow milk protein, and where breast milk is not an option
Specialized metabolic conditions where breast milk may not be an option	Carbohydrate-free formula	Infants with carbohydrate metabolism disorders and carbohydrate malabsorption
	Reduced and modified fat formula	Infants with fat malabsorption, chylothorax, and decreased bile salts
	Reduced mineral formula: lower phosphorus, iron, and potassium	Infants with calcium disorder, renal insufficiency

and Nutrition's (ESPGHAN) international expert group that was commissioned by The Codex Alimentarius Commission in November 2004 (100, 101).

There are several types of infant formulas (102, 103). Some have specific clinical indications for use, including special formulas for preterm infants, protein hydrolysate or elemental formulas for infants that have cow's milk and soy protein allergies, or formulas for other specific nutritional requirements. Other types of formula include indications such as lactose-free formulas for lactose-intolerant infants, soy formulas for galactosemia, and sensitive formulas that contain partially hydrolyzed or reduced lactose content (**Table 2**).

The development of infant formulas has advanced significantly over the past 50 years. Nonetheless, an "ideal" microbiome where *Bifidobacterium* species predominate cannot yet be obtained with infant formula feeding. Previously, we reported that *N*-glycans, which are released from cow's milk proteins, have prebiotic activity supporting the growth of *B. infantis* (90, 91). Thus, releasing *N*-glycans from proteins being added to infant formulas may be an innovative and effective strategy to harness the activity of naturally active enzymes in the microbiome of breastfed infants to enhance the bifidogenicity of infant formulas.

RELEASE OF *N*-GLYCANS FROM GLYCOPROTEINS

N-glycans can be released by chemical and enzymatic methods (105). However, enzymatic release is considered a preferred method as it eliminates the possibility of chemical or residual contamination. Moreover, due to the highly specific nature of the enzymes, the enzymatic release of *N*-glycans represents a more targeted and efficient approach for releasing and increasing the bioavailability of these bifidogenic structures. There are two known enzymes that can release *N*-glycans: *N*-acetylglucosaminidases and endo-β-*N*-acetylglucosaminidases (ENGases).

ENGases belong to EC number 3.2.1.X which corresponds to the glycosylase-type hydrolyses cleaving *O*- and *S*-glycosyl compounds. ENGases are further classified according to their glycoside hydrolase (GH) family membership. These enzymes are classified into two groups, GH families 18 and 85, based on their amino acid sequence (106) within the Carbohydrate-Active enZymes (CAZy) Database (http://www.cazy.org) (107). Family GH18 is unusual in having glycoside hydrolases that are both catalytically active chitinases and ENGases and also subfamilies of non-hydrolytic proteins that function as carbohydrate-binding modules/ "lectins" or as xylanase inhibitors whereas family GH85 solely contains ENGases.

Although all of the ENGases carry out the same hydrolytic reaction, they have different tolerances as to the precise structure of the *N*-glycans that they can hydrolyze. The ENGases are all retaining glycosidases that hydrolyze substrates via a two-step mechanism involving general acid/base catalysis. The main difference between GH18 and GH85 ENGases is the active-site amino acids either being two carboxylic acid residues (Glu and Asp) or one carboxylic acid and one amino group (Glu and Asn), respectively. Regardless of whether the active site contains one or two carboxylic acids, the hydrolytic mechanism catalyzed by the ENGases involves neighboring group participation of the 2-acetamide of the second GlcNAc residue (108).

ENGase enzymes cleave *N*-*N*′-diacetyl chitobiose moieties found in the *N*-glycan core of high mannose (HM), complex (CT), and hybrid (HY) *N*-glycans (**Figure 1**) and the released *N*-glycans that stimulate the growth of *B. infantis* (109) (**Figure 2**). EndoBI-1 from *B. infantis* (ATCC 15697) is a product of the Blon_2468 gene. Other *B. infantis* strains known to produce EndoBI-1 are JCM 7007, JCM 7009, JCM 7011, JCM 11346, ATCC 15702, and ATCC 17930 (110). The enzyme

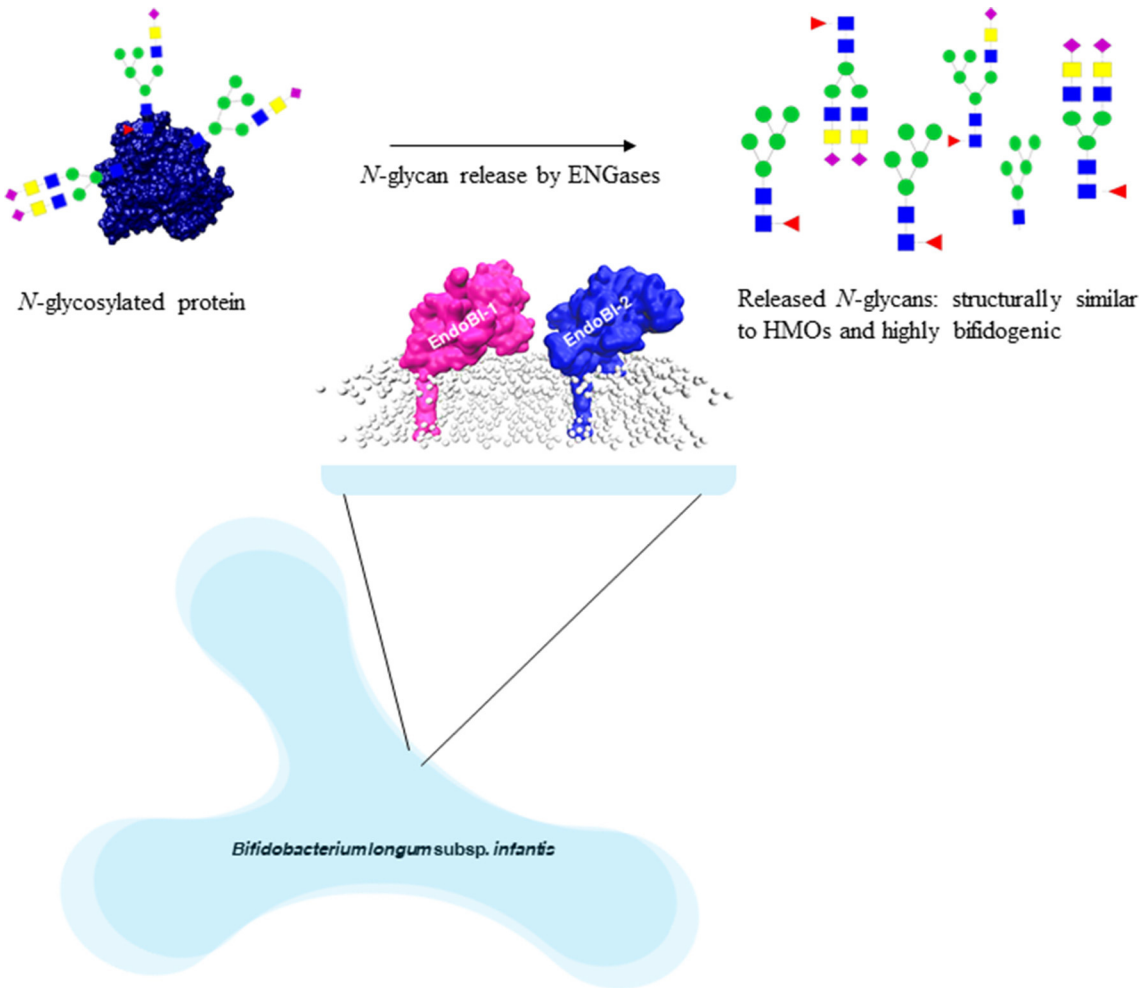

FIGURE 2 | Formation of highly bifidogenic *N*-glycans by *B. infantis* ENGases from glycoproteins (91, 92).

is classified as a GH20 member in the National Center for Biotechnology Information Genetic Sequence Database (NCBI-GenBank: ACJ53522.1) and EMBL European Bioinformatics Institute (EBI) European Nucleotide Archive (ENA CP001095.1) (111) and as a GH18 member in The Universal Protein Resource Knowledgebase (UniProtKB: B7GPC7) (110). The other ENGase, EndoBI-2 from *Bifidobacterium longum* subsp. *longum* 157F (deposited as *B. longum* subsp. *infantis* 157F), is a product of the BLIF_1310 gene (112, 113). The enzyme is classified as a GH18 member in NCBI-GenBank (BAJ71450.1). To date, only EndoBI-1 has been shown to be active in the gut of healthy breastfed infants colonized by *B. infantis* EVC001 (92), but both are likely to be expressed *in vivo*. Interestingly, EndoBI-1 and EndoBI-2 have different distributions among strains of *Bifidobacterium* found in infants compared to adults, which may further suggest the importance of these enzymes in healthy gut microbiome formation in both adults and infants (114).

EndoBI-1 and EndoBI-2 are unique among other ENGase members. EndoBI-1 and EndoBI-2 cleave *N*-glycans without perturbing the native glycan structure (115). The enzymes are considered fucose tolerant (110), meaning their activity is not affected by a fucosylated *N*-glycan core and therefore has a wider substrate specificity than similar enzymes (116). Both enzymes are active toward all major types of *N*-glycans found in glycosylated proteins (110). These unique enzymes are heat resistant, which enables broad applications even for industrial operations up to 95°C (110, 117), in contrast to the currently commercially available *N*-acetylglucosaminidases such as PNGase F of *Flavobacterium meningosepticum* which is heat labile (116). Further, both enzymes are considered safe for use in the food and pharmaceutical industries, especially when considering the sources of similar ENGase enzymes which are used by potential pathogens to evade the host immune system; such as Endo-COM from *Cordyceps militaris* (118), EndoS and EndoS2 from *Streptococcus pyogenes* (119, 120), EndoF3 from *Elizabethkingia meningoseptica* (121, 122), EndoH from *Streptomyces plicatus* (123, 124), EndoD from *Streptococcus pneumoniae* (3GDB.pdb), and EndoT from *Hypocrea jecorina*

(125). Thus, making EndoBI-1 and EndoBI-2 the only two enzymes currently considered safe for food applications. Importantly, EndoBI-1 and EndoBI-2 could be easily cloned and/or mass produced with known microbiologic procedures and industrial techniques (110).

CHALLENGES IN THE STUDY AND CHARACTERIZATION OF *N*-GLYCANS

One of the primary challenges facing the translation of technologies surrounding *N*-glycan release is the precise and accurate quantification and characterization of *N*-glycans. Structural analyses of oligosaccharides and glycoconjugates by high-throughput approaches are crucial for predicting their functions. A number of chromatographic techniques have been employed for the analysis of oligosaccharides (126). One of the most common is porous graphitized carbon chromatography–mass spectrometry (PGC-MS) (127). This method can distinguish the isomers of oligosaccharides and *N*- and *O*-glycans of glycoconjugates with different linkage positions. This ability of PGC-MS makes the method more powerful than previous techniques. To achieve the structural identifications of HMOs faster and with more precision, a library was recently presented for both native and sialylated oligosaccharides, including retention times, accurate masses, and tandem mass spectra of HMOs (38, 42). In addition, relative and absolute quantification of HMOs was performed using the PGC-MS approach (128). Thus, the alterations of HMO profiles could be monitored throughout certain periods such as lactation. For example, a specific method was recently demonstrated for the absolute quantification of neutral and acidic HMOs (129). PGC-MS can also be used for the characterization of glycoconjugates of human milk. In one study, *N*-glycans released from human milk whey glycoproteins were analyzed and compared with bovine milk *N*-glycans using the PGC-MS technique (130). On the other hand, a method including solid-phase permethylation step was presented for the analysis of HMOs and glycans derived from human and bovine milk whey glycoproteins by reverse-phase liquid chromatography mass spectrometry (RPLC-MS) (131). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-MS) has also been commonly used for the characterization of HMOs and glycoconjugates (132, 133). This approach makes the analysis very fast when compared with chromatographic and electrophoretic techniques. In this approach, typically neutral HMOs and *N*-/*O*-glycans can be quantified using MALDI-MS because of unstable sialic acid residues found in HMOs and glycoconjugates. However, sialic acids can be derivatized by certain methods to make them more stable during the MALDI-MS analysis (134).

POTENTIAL APPLICATION OF ENGASES IN NEXT-GENERATION FORMULAS AND CHALLENGES

Although the composition of human milk is unparalleled in terms of suitability for infant nutrition, there are a number of logistical, practical, and medical reasons that necessitate the use for infant formulas with barriers to breastfeeding and racial inequities and/or socioeconomic barriers being prominent (135–138). The use of microbial enzymes is a staple of the industrial progress in the 21st century (139). The development of infant formula has not been the exception. For example, various next-generation infant formulas have been developed to manage cow milk protein allergy. Infant formulas with reduced allergenicity generally have partially or extensively hydrolyzed proteins, or amino acid-based formulations. Allergenicity is decreased by converting proteins to smaller peptides for modifying conformation or structure epitopes recognized by the immune system while maintaining caloric and protein and content, or by replacing intact proteins or peptides with amino acid formulations (140).

The process of producing partially or extensively hydrolyzed proteins involves complex proteolytic processing steps to reduce the size of bovine milk proteins. Protein glycosylation provides a stabilizing effect to proteins, making the native protein state more resistant to degradation (141, 142). Glycosylated proteins are more resistant to proteases compared to their aglycosylated (never glycosylated) or deglycosylated (enzymatically removed) counterparts (143–145). In fact, the rate of proteolysis and the amount of intact peptide (epitopes) available to reach up the intestinal tract are influenced by the presence of structural glycans (146). As bovine milk protein processing represents a major hurdle for the production of partially and extensively hydrolyzed proteins in infant formulas, the introduction of ENGases to this process has the potential to increase the efficiency and extent of protein hydrolysis required for infant formulas.

Deglycosylation may also have implication for bioactive proteins and the released *N*-glycans. For instance, lactoferrin, an important bioactive protein added to formula, is heavily glycosylated. Modifying glycosylation patterns is likely to change bioactive sites and catalytic activities (147). Further, the released *N*-glycans from glycoproteins can be recovered from protein production streams and used as an added source of highly specific prebiotics for the infant gut microbiome. These *N*-glycans are then converted into metabolites with energy value for the infant (e.g., acetate and lactate) when competent *Bifidobacterium* are present, as well as to enhance the colonization of specialized bifidobacteria, such as *B. infantis*, which provide essential ecosystem services to the infant gut (**Figure 3**) (94).

The ENGase enzymes may be either used in the production step to release high *N*-glycan concentrations in the infant formulations or included as components of designed formulas to release *N*-glycans *in situ* in the gut. Theoretically both native ENGases produced by baby and infant safe organisms such as EndoBI enzymes of *B. infantis* and ENGases produced by recombinant organisms could be used for these applications. However, in practice regulations in most of the globe and especially in EU do not allow the use of GMO in baby and infant products.

Although baby food and infant formula prepared with ENGases produced by recombinant organisms used in the production step could be considered as products derived with

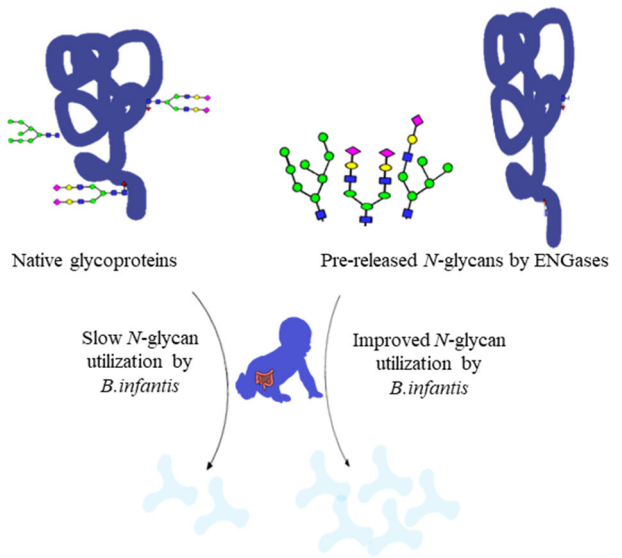

FIGURE 3 | Utilization rate of native glycoproteins and pre-released glycans by ENGases (91).

GMOs, public and private standards for baby and infant products are too strict to use these products yet for both US and EU markets. Therefore, in the immediate future instead of ENGases produced by recombinant organisms, ENGases produced by baby and infant safe organisms such as EndoBI enzymes of *B. infantis* are more realistic. Although the regulators in the European Union can change the complete regulatory system from a process-based system to a strictly product-based system, such as in Canada in the future, these changes are unlikely to affect baby and infant products.

CONCLUSION

The use of ENGase enzymes in the production of infant formula has great potential to increase the nutritional values of formula by releasing additional carbohydrates as sources of energy and substrates from *N*-glycans, a so far underexploited and underappreciated source. Due to their structural similarity to the HMOs, the release of *N*-glycans is likely to be a more successful approach to increase the potential for infant formula to promote colonization of the infant gut by infant-adapted *Bifidobacterium*, leveraging ingredients already present in these formulations and a growing understanding of the microbial enzymes active in the infant gut ecosystem. Finally, deglycosylation of proteins also has the potential to create value-added formulations as well as to have implications on a manufacturing scale.

AUTHOR CONTRIBUTIONS

SK organized the general content of the paper. HD was responsible for general editing and organizing the authors as well as the contribution for two sections. MK, AS, and HK contributed one section of the paper. AA and SF were responsible for one section of the paper. All authors contributed to the article and approved the submitted version.

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Heat Treatment of Milk: A Rapid Review of the Impacts on Postprandial Protein and Lipid Kinetics in Human Adults

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Background: Most milk consumed by humans undergoes heat treatment to ensure microbiological safety and extend shelf life. Although heat treatment impacts the structure and physiochemical properties of milk, effects on nutrient absorption in humans are unclear. Therefore, a rapid review was performed to identify studies conducted on healthy human adult subjects that have assessed the impacts of heat treatment of milk on protein and fat digestion and metabolism in the postprandial period (up to 24 h).

Methods: Relevant databases (Medline, EMBASE, Cochrane, Scopus) were systematically screened for intervention studies on healthy adult men and women that assessed the impact of consuming heat-treated milk on the postprandial kinetics or appearance in peripheral circulation or urine of ingested proteins and/or lipids. The risk-of-bias assessment tool 2 was used for quality assessment.

Results: Of 511 unique database records, 4 studies were included encompassing 6 study treatments (n = 57 participants, 20–68 years). Three studies evaluated pasteurization, two evaluated ultra-high temperature (UHT) treatment, and one evaluated oven-heated milk. Protein and lipid appearances in peripheral blood were reported in two sets of two studies. None of the studies used the same heat treatments and outcome measures, limiting generalization of effects. Protein appearance (ng/mL or area under the curve) (as plasma amino acids - lysine) was reduced when milk was oven-heated for 5 h in one study (n = 7 participants), while the other study reported a reduced retention of dietary N with UHT milk (n = 25 participants). Overall plasma triacylglycerol responses were unaffected by milk heat treatments reported, but plasma fatty acid composition differed. The studies observed higher plasma myristic and palmitic acid abundance with successive heat treatment at 2 h (n = 11 participants; pasteurized) and 4 h (n = 14 participants; UHT) after ingestion; other differences were inconsistent. All studies had moderate-high risk of bias, which should be taken into consideration when interpreting findings.

Discussion: This review identified few studies reporting the effects of milk heat treatment on postprandial nutrient responses in adults. Although the findings suggest that milk

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heat treatment likely affects postprandial protein and lipid dynamics, generalization of the findings is limited as treatments, outcomes, and methods differed across studies. Because of the study variability, and the acute post-prandial nature of the studies, it is also difficult to draw conclusions regarding potential long-term health outcomes. However, the possibility that altered digestive kinetics may influence postprandial protein retention and anabolic use of dietary N suggests heat treatment of milk may impact outcomes such as long-term maintenance of muscle mass.

Keywords: lipid, protein, postprandial, dairy, pasteurized, ultra-high temperature

INTRODUCTION

Heat treatment is a widely used technique in the dairy industry, as fresh liquid milk is a product with a short shelf-life. In addition, although the careful use of animal husbandry and on-farm hygiene practices enables raw milk of high microbiological quality to be obtained, raw milk can be microbiologically unsafe for human consumption (1, 2), due to the presence of harmful bacteria including *Campylobacter*, *Salmonella*, Shiga toxin-producing *Escherichia coli* (3) and *Listeria monocytogenes* (1, 2). Heat treatment has been defined as follows by the International Dairy Federation: "Any intentional heating above 50 °C for a sufficient time such that there is a reduction in the concentration of one or more microorganisms is considered heat treatment" (4). Standard heat treatment includes high-temperature short time pasteurization (72 to 80 °C for 15 to 30 s) and ultra-high temperature (UHT) processing (135 to 150 °C for 1 to 10 s) (4), with the primary objective to make milk safe for human consumption.

With a view to understanding the impact of milk processing on human health, investigations of the consequences of processing on milk structure and physical properties have been conducted using *in vitro* systems (5–7). Pasteurization and UHT processing were shown to increase the aggregation of fat and protein into a semisolid curd under simulated gastric conditions (5). UHT milk was shown to have faster rates of protein hydrolysis compared to pasteurized milk and a faster release of fat globules during digestion. Those results were explained by differences in the structure of the different milk curds (6). Protein modifications occurring during heating processes (i.e., pasteurization) have been shown to impact protein composition and function of the milk fat globule membrane, which have the potential to impact digestion (8). Those structural changes, especially in UHT milk, include a looser curd structure, creating a larger surface area for the diffusion of pepsin into the curd (7). A smaller weight of milk curd can lead to a quicker protein hydrolysis and thus, speed up the gastric emptying process (6). Moreover, a rodent study assessing the impact of different thermal treatments (UHT, pasteurized, and spray-dried milks) on the bioavailability of dairy proteins showed a modification of the postprandial splanchnic protein extraction and a small but significant impairment in digestibility following spray-dried milk, with no impact of heat treatment on nitrogen availability. The authors concluded that altered postprandial metabolism may relate to the degree of protein lactosylation (9); lactosylation is enhanced by heat treatment (10).

However, the impact of heat treatment on the ways in which proteins and lipids are absorbed in humans have not been detailed by these *in vitro* or animal studies. Our objective was to identify studies conducted on healthy human adult subjects that have assessed the impacts of heat treatment of milk on protein and fat digestion in the postprandial period (up to 24 h), using non heat-treated milk as a comparison across studies where possible. For this purpose, we used a rapid review methodology to review the literature.

METHODS

A rapid review methodology was used to complete this study. The objective of a rapid review is to synthesize knowledge in order to produce information under time constraints by simplifying the systematic review process. Systematic reviews usually take many months, or even up to years to produce, whereas rapid reviews typically only take from 1–6 months, and may limit the scope of sources searched (e.g., gray literature) (11, 12).

Using the Population Intervention Comparator Outcome Time (PICOT) format (13), our research question was formulated as follows: Are there differences in protein and/or lipid digestion and metabolism in the 24 h following consumption of different types of heat-treated milk, or heat-treated compared to non-heat-treated milk, among adult men and women (18–70 years)? (**Table 1**).

TABLE 1 | PICOT criteria employed to define the research question.

Criteria	Description
Participants	Human men and women (18–70 years)
Intervention	Heat-treated milk
Comparison group	Non-heat-treated milk, or milk undergoing a different heat treatment
Outcome of interest	Parameters of protein and/or lipid digestion or metabolism
Time	$\leq$ 24 h following ingestion of milk

TABLE 2 | Inclusion and exclusion criteria applied in article screening.

	Inclusion	Exclusion
Population	Healthy human participants Men and women Adults (18–70 years)	Animals Population with chronic diseases (e.g., cardiovascular diseases, cancer) metabolism disorders (e.g., thyroid) or gastrointestinal diseases (e.g., celiac disease, inflammatory bowel disease) Populations with known intolerance to milk
Study design	Interventional Follow up maximum 24 h	Observational Study cases Interventions over 24 h long
Study setting	Clinical trial	Community
Intervention	Consumption of a meal with milk that was heat treated (e.g. UHT, pasteurization, ESL)[1]	If milk was not heat treated and only went through another type of process (e.g., microfiltration, homogenization)
Outcome	protein and/or lipid kinetics; protein and/or lipid appearance and clearance in blood circulation and/or urine	
Publication status		Full text is not available in English

According to the International Dairy Federation, "Any intentional heating above 50° C for a sufficient time such that there is a reduction in the concentration of one or more microorganisms is considered heat treatment" (4). ESL, extended shelf life; UHT, ultra-high temperature.

Inclusion Criteria

The inclusion criteria (**Table 2**) were determined by one reviewer (MF) and then shared for approval with the second reviewer (AM).

Search Strategy

The electronic databases Medline (ovid), EMBASE (ovid/ 1980-present), The Cochrane Library and Scopus were searched without any restrictions to the time of publication. The search was carried out from June 10–16th 2020. The research question was separated in four concepts grouping all the Medical Subject Headings (MeSH) and keywords relevant to each concept. All those terms were linked within each concept with the connector OR and the four concepts were joined using the connector AND. The MeSH terms were adapted according to the specificity of each database. No MeSH terms were used to search Scopus but only the keywords in the title, abstract and keywords of the database. The full search strategy and its adaptation for each database can be found in **Supplemental Tables 1–4**.

All the search results were exported to the reference manager Mendeley (v1.19.4 Elsevier Inc., Amsterdam, Netherlands) where duplicate articles were removed.

Screening Process

Titles and abstracts of articles were screened independently by two reviewers (MF and AM) against the inclusion and exclusion criteria. The discrepancies were resolved through discussion, or by consultation with a third reviewer if necessary (NG). If the decision to include an article was unclear at any stage, then the full text was obtained. Next, the full text of relevant articles was obtained and screened to ensure their eligibility.

Data Extraction

The two reviewers independently extracted the following information from the relevant full-text articles: author, date and country of the article, purpose of the study, type of heat treatment, temperature and length of the treatment, milk of comparison, volume of milk, time of follow-up, number, sex and age range of the participants, outcome measurement and results.

Quality Assessment

The risk of bias was assessed using the Cochrane risk-of-bias assessment tool for randomized trials (RoB 2) (14). The risk of bias was assessed (by MF) for each independent outcome within a study where differences between the outcomes were observed (e.g., missing data for a specific outcome, different number of analyzed samples).

RESULTS

Study Selection

A total of 1,076 articles were identified from the four database searches and exported to Mendeley. The duplicate check was then completed, resulting in 511 articles. After the title and abstract screening, 506 articles did not match the inclusion criteria and were excluded from full-text review. Finally, we assessed the full-text of 5 articles, from which 1 was excluded because the measured outcomes did not meet the inclusion criteria (**Figure 1**).

Study Characteristics

Two of the studies were conducted in Finland by the same group (15, 16). One was conducted in France (17), and one in Sweden (18). One study was a randomized parallel trial (17) and the other three studies were randomized cross-over trials (15, 16, 18). In total, $n = 57$ subjects were included. All studies were conducted on both male and female subjects. Most subjects were aged from 20 to 40 years old but one study reported their eldest subject to be 68 (15). All the subjects were reportedly healthy. In two of the studies (15, 16), subjects reported gastrointestinal (GI) symptoms after drinking milk but no lactose intolerance had been previously diagnosed. Across the four studies, there were six treatment groups in which heat-treated milks were consumed. Three studies measured the effects of pasteurization (15–17), two measured the effects of UHT treatment (16, 17) and in one study the milk was heated in an oven (18). Measured outcomes were glycemia in three of the studies (15–17), insulinemia, lipemia, inflammation and GI symptoms in two of the studies (15, 16), amino acids (AAs) in plasma in one study (18) and AAs, protein and urea in serum, urinary creatinine, urinary urea and dietary N incorporation in one study (17) (**Table 3**).

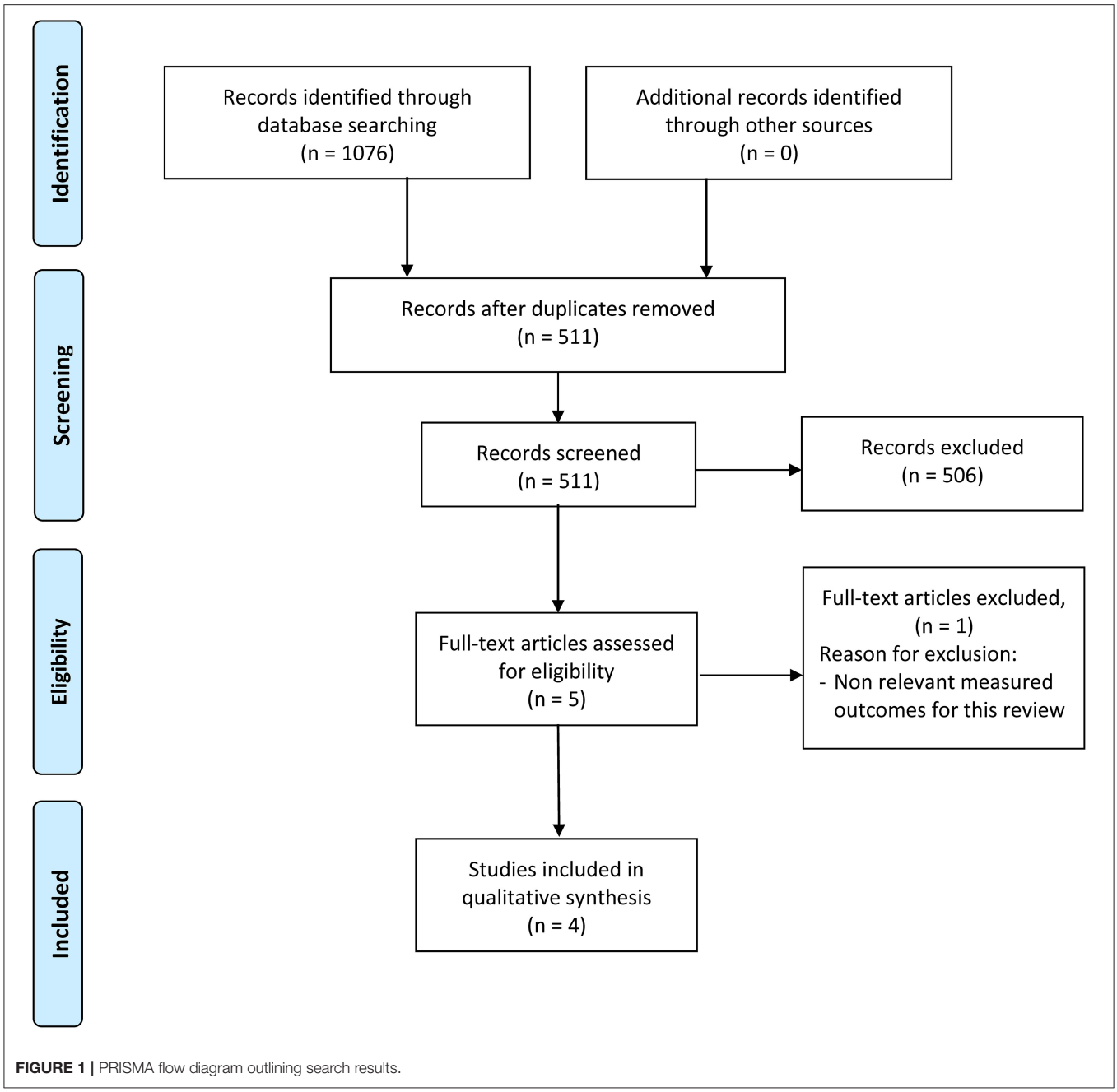

FIGURE 1 | PRISMA flow diagram outlining search results.

Risk of Bias Within Studies

Three of the studies were assessed with some concerns on the overall bias (15–17) and one was assessed with a high risk of bias (18).

These findings reflected a lack of reporting within the methods used in the study by the investigator team (e.g., lack of reporting of a randomization strategy or a pre-specified analysis plan before having the results). For instance, pre-specified analysis plans were not specified or available for all studies, meaning that none of the included studies could satisfy a low risk of bias under Domain 5 of the tool. No differences in risk assessment were found within studies reporting multiple outcomes, so risk of bias has been presented only once per study (**Figure 2**).

Summary of Study Findings

Proteins and Amino Acids

The study that measured the plasma AA response 2 h after ingestion of milk heated in the oven [Ljungqvist et al. (18)] found a significant drop in the lysine plasma concentration between the milks heated for 0 h (−3.9%) and 5 h (−10.1%); hence, compared to raw milk (0 h) the lysine plasma concentration was lower. However, the study by Lacroix et al. that assessed the plasma AA

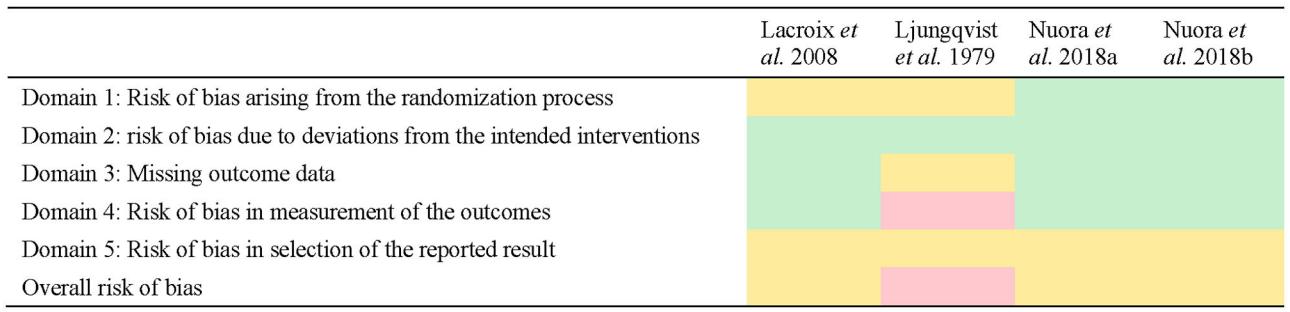

FIGURE 2 | Quality assessment of studies using the Risk of Bias 2 tool. Green indicates low risk, yellow indicates some concerns, red indicates high risk.

concentrations after the ingestion of micro filtered milk (MF), pasteurized milk (PM) and UHT-treated milk (17) did not report any significant differences across plasma AA, including lysine plasma concentration. Lacroix et al. also reported significant differences between the time courses of dietary N transfer into serum AA, urea N, and protein pools, using ^{15}N milk labeling. UHT showed 5% higher dietary N in the body urea pool after 8 h than MF and PM; in serum proteins this elevation was ∼1% higher. A significant meal effect was observed after the consumption of UHT milk compared to PM and MF milk (**Table 4**). A postprandial reduction in dietary N retention of 8% was also observed after ingestion of the UHT compared to the two other types of milk (**Table 4**).

Lipids

No significant differences were found in the plasma triacylglycerol concentrations between homogenized, pasteurized and UHT milk (16), nor in the blood triacylglycerol concentrations between raw milk ("native milk"; NM) and homogenized and pasteurized milk (HPM) (15). However, significant differences were found in the fatty acid composition of plasma lipids. Myristic, palmitic, and stearic acids were found to be higher 4 h after the consumption of the HPM compared to the NM. However, no significant differences were observed at the 2 h time point (15). In the other study measuring this outcome (16), significant differences were also reported. At the 2 h time point, myristic, palmitic, oleic and linoleic acids were found to be significatively higher after the UHT milk and the PM compared to the HPM. At the 4 h time point, linoleic acids levels were found to be higher after PM compared to the UHT milk that was itself higher than the HPM.

Plasma Glucose and Insulinemia

The three papers that measured plasma glucose concentration after drinking the milks did not report any significant differences between milks processed by different methods regarding that outcome (15–17). No significant differences were reported for plasma insulin levels either (15, 16).

DISCUSSION

The purpose of this review was to identify studies that assessed the impact of heat treatment of liquid milk on protein and lipid digestion and metabolism in human adults. A total of four papers reporting studies on healthy human subjects were included, of the 511 initially identified. All studies were assessed as having moderate-high risk of bias. Two of the studies reported outcomes related to lipids (15, 16) and the other two reported outcomes related to proteins (17, 18). Despite the topic of milk heat treatment being relevant for commercial milk products (3) and consumer nutritional preferences (19, 20), we did not find many studies measuring our outcomes of interest (i.e., protein and fat metabolism after consumption) directly in human subjects.

Our search resulted in 511 articles to screen, although only four articles met the inclusion criteria, all of which described studies that were conducted in Europe. Most were excluded for reasons such as being conducted on animals (9, 21, 22) or infants (23–25), only assessing physical and technological milk properties (26) (e.g., rheological and thermal properties, microstructure), participants drinking milk protein mix (e.g., whey beverages) but not plain milk (27, 28), studies based on plant-based milk (29, 30), or measured outcomes which were not relevant for this review (e.g., vitamins, iron, zinc) (31). Indeed, a recent systematic review of the processing impacts on milk protein digestion (32) identified only two human studies meeting their criteria, of which only one [Lacroix et al. (17)] investigated liquid milk (in this case, defatted) and is therefore common to our comparison. The large number of irrelevant articles may have been avoided with refinement of search terms; however, the current search strategy also highlighted areas which may be suitable for future reviews into the effects of heat treatment of milk on digestion in contexts other than liquid milk or human populations. As only four studies were included in the final assessment, this suggests either a lack of studies conducted specifically on adult human subjects for the specified outcomes or a lack of additional search terms that could be relevant. For instance, terms such as *in vivo*, nitrogen metabolism, or alternate terminology for heat treatments may have been missed, in addition to possible content available in the gray literature.

Half of the studies were conducted on self-described milk intolerant people but with no clinically diagnosed intolerance

TABLE 3 | Characteristics of included studies assessing postprandial protein or lipid responses to heat treated milk in adults.

		Lacroix et al. (2018) (17)	Ljungqvist et al. (1979) (18)	Nuora et al. (2018a) (15)	Nuora et al. (2018b) (16)
Study characteristics	Country	France	Sweden	Finland	Finland
	Study objective	To assess impact of heat treatment (microfiltration, Pasteurization, UHT) on protein quality (measured by nitrogen metabolism) following single meal	To assess impact of lactose hydrolysis of skim milk powder on lysine availability (in heat treated samples)	To assess impact of native milk v homogenized & pasteurized milk on gastrointestinal symptoms, inflammation, transit, intestinal pressure, glycemia, insulinemia or lipemia	To assess impact of milk processing on gastrointestinal symptoms, inflammation, lipemia, glycemia, insulinemia
Participant characteristics	*n*	25 (11M; 14F)	7 (3M; 4F)	11 (5M; 6F)	14 (6M; 8F)
	% male	44%	43%	45%	43%
	Age range (years)	Age range not reported, range of means: 23.5 ± 6.9–27.1 ± 7.8	24–29	24–68	20–45
Heat treatment	Type	1. Pasteurized 2. UHT	Lactose hydrolyzed freeze-dried skim milk heated in oven	Homogenized pasteurized	1. Homogenized pasteurized. 2. Homogenized UHT
	Temperature	Pasteurization: 72 °C; UHT: 140°C	66 °C	72–73 °C	Homogenized Pasteurization: 73 °C; Homogenized UHT: 135 °C
	Length of treatment	Pasteurization: 20 s; UHT: 5 s	1, 3, and 5h	15 s	Homogenized Pasteurization: 15 s; UHT: 3 s
Comparison milk		Microfiltered	Lactose hydrolyzed freeze-dried skim milk without oven-heat treatment	Raw milk	Pasteurized[a]
Intervention	Study protocol prior to testing	Standardized diet, adjusted to participant's body weight, was provided for one-week prior to the study day. Overnight fast prior to testing.	Overnight fast prior to testing.	Non-dairy diet for 5 days prior to the study day. Overnight fast prior to testing.	Non-dairy diet for 5 days prior to the study day. Overnight fast prior to testing.
	Test meal	No test meal consumed with the milk.	Milk samples were mixed with gluten (1:1 on basis of protein content)	SmartBar (Given Imagine, Israel)	Rice cakes (24g), turkey cold cuts (85g), cucumber (50g)
	Volume of milk consumed	500 mL	400 mL	400 mL	400 mL
	Duration of follow-up	8 h	2 h	4 h	5 h
	Frequency of sampling	30, 60, 90, 120, 150, 180, 240, 300, 360, 420, and 480 min after ingestion.	2h after ingestion.	20, 40, 60, 90, 120, 180, and 240 min after ingestion.	20, 40, 60, 90, 120, 180, 240, and 300 min after ingestion.
Milk characteristics	Fat content - test	Defatted milk	Not reported	Not reported	Not reported
	Fat content - control	Defatted milk	Not reported	Not reported	Not reported
	Protein content - test	23.3g	Not reported	34.6 ± 0.6 g/kg	Not reported
	Protein content - control	23.3g	Not reported	34.8 ± 1.7 g/kg	Not reported

[a] *Pasteurized milk was heated under the same conditions as the homogenized pasteurized milk, but did not undergo earlier homogenization at 16 MPa before heat treatment. F, female; UHT, ultra-high temperature.*

TABLE 4 | Findings of included studies assessing postprandial protein or lipid responses to heat treated milk in adults.

	Lacroix et al. 2008	Ljungqvist et al. 1979	Nuora et al. 2018a	Nuora et al. 2018b
Outcome measurements	Blood glucose serum urea, AA, protein, N urinary creatinine & urea, ammonia, N N incorporation into body pools	Plasma AA% molar ratio of AA	Gastrointestinal symptoms, inflammation, transit, intestinal pressure, glycemia, insulinemia, lipemia/fatty acids	Gastrointestinal symptoms, inflammation, lipemia, glycemia, insulinemia
Significant results	Dietary N: body urea: 15% UHT v 10% MF/PM serum proteins: 7.7 ± 1.2% UHT v 6.1 ± 1.0% MF v 6.4 ± 1.5% PM urinary urea: 11.7 ± 3.1% UHT v 8.0 ± 2.1% MF v 8.1 ± 2.4% PM 8 h in body urea: 25.9 ± 3.3% UHT v 18.5 ± 3.0% MF v 18.6 ± 3.7% PM NPPU: MF = PM, UHT lower by 8% UHT greater N loss	Limited plasma lysine & sulfur AA in all heat-treated milk v unheated: lysine plasma AA at −3.9 at 0 h and at −10.1 at 5 h	Higher plasma myristic, palmitic, stearic acid 4 h after HPM than NM	Higher plasma myristic, palmitic, oleic, linoleic 2 h after UHT or PM than HPM linoleic higher PM than UHT, also UHT higher than HPM at 4 h
Quality assessment	Some concerns	High risk	Some concerns	Some concerns

AA, amino acids; F, female HPM, homogenized pasteurized milk; MF, microfiltered milk; M, male; N, nitrogen; NM, native milk; NPPU, net postprandial protein utilization PM, pasteurized milk; UHT, ultra-high temperature.

(15, 16). GI symptoms were indeed described by study participants. It is unclear whether the inclusion of participants who experience symptoms such as bloating or cramping may have influenced the outcome measures of digestion, and it is not possible to compare and contrast evidence due to the small number of studies conducted. We did not take into consideration the reported outcomes of those studies regarding GI symptoms and inflammation markers as they were not outcomes of interest for this review. Although there is some evidence that plasma AA appearance after milk ingestion is unimpaired in subjects with lactose intolerance, other forms of dairy intolerance may impact plasma AA (33) or other micronutrient responses (34). Conducting studies on subjects that do not experience GI symptoms would be the next step in getting a better understanding of how heat processing influences the composition of circulating fatty acids, as both studies evaluated in this review included subjects with reported GI symptoms.

Only one study assessed outcomes related to protein metabolism (17) with this study also investigating serum AA dynamics. This showed that protein digestion may be accelerated with heat treatment, in this case UHT, with this change in digestive dynamics potentially driving enhanced anabolic use of dietary N specifically in serum proteins. However, this study also showed that net postprandial protein utilization (NPPU) was significantly reduced in the UHT group, indicating that more nitrogen/amino acids were oxidized in the UHT treatment. This suggests that overall protein retention was lower, and on a whole-body level the lower NPPU of the UHT treatment group reflects a reduced anabolic effect. One other study also assessed AA dynamics in plasma (18). In this case, AA concentrations at 2 h were lower with heat-treated milk (oven heated) *vs*. raw milk, suggesting reduced AA availability after heat treatment. However, it is also possible that the lower AA concentrations after heat treatment may reflect more rapid clearance from plasma and use for protein synthesis. The impacts of heat treatment on protein digestion have been assessed *in vitro* (6) and in animals (9, 21, 35), and indeed, many studies that were excluded from the review assessed protein digestion yet in the context of derivatives of liquid milk (36) or in non-adult populations (23, 24). Yet, few studies have measured protein or amino acid dynamics in human adult subjects. Given the large body of evidence to support protein modifications to dairy products *in vitro* and in animal models [as reviewed by van Lieshout et al. (32)], it is perhaps surprising so few human studies exist.

The quality assessment indicated that some concerns on the risk of bias existed in three of the studies (15–17) with one study having a high risk of bias (18). The study by Ljungqvist et al. (18) was considered to have a high risk of bias primarily because the number of samples included in the results did not always match the number of participants, nor were they consistent at each time point, and no explanation was provided for this discrepancy. The study design and implementation was also unclear (e.g., no information is provided about the randomization of the sequence, if the trial is blind or double-blind) and may in part be explained by the time the study was conducted (c. 1979) relative to the introduction of standard reporting guidelines such as Consolidated Standards of Reporting Trials (CONSORT) (37). Indeed, most of the studies may have had higher quality assessment scores if the research methodology had been reported more thoroughly. In many cases, domains were scored with at least some concerns on the risk of bias because information was missing (e.g., a clear explanation on how the randomization sequence was obtained, no indication of whether the outcomes were measured on all the participants, reporting of the subject characteristics). Taking the RoB 2 tool guidelines (14) into account when determining the design of a randomized trial and then when reporting the methodology would help to avoid any doubts on the reliability of the trial's results. The quality of the three most recent papers (15–17) appears to be satisfactory in their lower risk of bias to provide a reliable quality of evidence due to their detailed reporting of the milk characteristics, inclusion and exclusion criteria, experimental design and methods of analysis of the outcomes.

The overall findings suggest significant differences in the plasma fatty acid composition due to milk processing methods, including greater plasma concentrations of myristic and palmitic acids after UHT relative to HPM (16), and relative to NM (raw milk) (15). However, it is important to note that the two studies assessing this outcome (15, 16) were conducted by the same group of researchers so similarities in methodology used could contribute to the alignment of these findings. Moreover, each study made comparisons between different types of milk processing, so it is not possible to determine whether consistent results have been observed across studies for any specific milk processing type. Subjects also did not have their diet standardized, apart from not consuming dairy products 5 days before the trial. Participants of both studies also experienced GI symptoms after milk consumption such as cramping or bloating (15, 16). It is possible that these symptoms could influence the findings of nutrient appearance in blood circulation. There is evidence that GI symptoms (38, 39), as well as GI disorders including inflammatory bowel disease, Crohn's disease and celiac disease (40–42) are associated with reduced absorption of some nutrients. GI symptoms such as diarrhea or constipation are also known to be characterized by changes to GI transit times (43); however, the extent to which these symptoms impact small intestinal absorption, or whether these factors influences any of the reported findings is not clear. These studies were not able to determine the mechanisms for blood circulating fatty acid composition differences at specific time points following consumption of processed milks. It is still unclear how the lipid droplet size differences observed after *in vitro* digestion of pasteurized cream (22) influences the intestinal absorption of lipids. Therefore, conducting a study on a bigger group of people, standardizing diets prior the trial, ensuring subject homogeneity, etc. could help to limit variability and remove some of the sources of possible uncertainties that could influence the results.

Regarding measures of protein digestion and metabolism, results were not conclusive due to the limited number of studies available, and the variation in outcome measures used. Yet, both studies reported findings on both sexes, of similar ages (∼20–30 years), and consuming similar quantities (400 vs. 500 mL). The study with a high risk of bias, non-commercial heating methods, and shorter follow-up (2 h) (18) reported a significant loss in the plasma lysine concentration with milk heated for a longer period of time, but those results did not match a more recent study with a better quality assessment, standard processing (i.e., PM and UHT) and longer follow-up (8 h) (17) that reported no significant changes in the serum AAs or in the lysine serum concentration with UHT milk relative to MF or PM. The latter study also provided a separated analysis of dispensable (non-essential) AAs, indispensable (essential) AAs and lysine concentration in the serum, although, no further information about the AA composition in serum was provided. Further, the heat treatment of the milks between studies differed: oven heated at 66°C for 1–5 h [lower temperature and longer duration than standard processing methods (32, 44)] vs. UHT, pasteurized and homogenized milk. These differences limit the ability to compile the effects of any one type of heat treatment on AA appearance, and in terms of relevance to commonly consumed products, data were available from only a single study.

This review highlights the current lack of studies investigating the impacts of milk heat treatment on protein and lipid digestion involving human adult participants. *In vitro* studies have been able to demonstrate that processing affects milk structure and thus digestion by changing the rates of protein hydrolysis and the release of milk fat (5–7, 32) but this is not yet the case for *in vivo* studies on humans. Variability in methodologies used further complicates the ability to draw consensus conclusions across the studies that were identified. Across studies, the milks underwent various heat treatment methods, with few comparable comparisons between the studies. One study compared UHT milk and pasteurized and homogenized milk with pasteurized milk only (16); no non-heated milk was used as a control. Three other studies used non-heated milk as a control; however, different processing techniques were still used. One study used raw milk (as NM) (15), another microfiltered milk (17) and one study used lactose hydrolyzed freeze-dried skim milk (18). Indeed, not having the same control makes it more difficult to compare findings and provide confident summaries. Likewise, the methods used to assess study outcomes, particularly protein and amino acid kinetics, were diverse. Only one study measured dietary N transfer and showed that UHT processing reduced this outcome compared to pasteurization and microfiltration (17). While this suggests heat treatment may have implications for long-term health outcomes relative to protein utilization, none of the other studies included in this review assessed this outcome so further investigation is required to clarify the impact that the different heat treatments have on the use and retention of ingested milk proteins.

This review has a number of strengths. The objective of the study was developed based on an established methodology (PICOT) and the search is well-described and rigorous, including a large amount of words and terms relevant for the objective. In addition, two of the authors did individual screening of the search results. A quality assessment was performed using the Cochrane risk-of-bias assessment tool for randomized trials, to provide an indication of the quality of research on which any conclusions were based. Finally, we have undertaken a critical discussion of the findings.

We acknowledge several limitations of the review. First and foremost, very few relevant studies were found, and the number of study subjects was relatively small, making it difficult to draw any clear conclusions. However, we believe this is in itself interesting, as it demonstrates that relatively little research has addressed the posed question, and this therefore clearly indicates that further research is required. It is also possible that content available in the gray literature may have proven useful, although given the nature of this rapid review a conscious decision was made to exclude such literature.

Because of the small number of studies identified, and the range of different heat treatment regimes used in these studies, it was also not possible to make a meaningful comparison of particular heat treatments, and we also note this as a limitation.

Although we believe this was an extensive search, some key words that could be considered relevant were not included, for example "apoB-48" and "chylomicrons" which are relevant to lipid digestion. However, we note that a

subsequent search including these terms did not identify any additional studies.

CONCLUSION

The present review showed an overall shortage of studies conducted on this topic on adult human subjects; as such, no solid conclusions about the impact of heat treatment of milk on protein and lipid digestion can be made. Differences between types of milk heat treatment could be shown on plasma fatty acid composition by the different articles but limitations to those findings prevented any conclusion about the way lipids and fatty acids are metabolized. No conclusive results could be obtained about how heat treatment affects postprandial protein metabolism. The lack of conclusive findings regarding both lipid and protein metabolism also makes it difficult to draw conclusions regarding long-term health impacts of consuming milks undergoing different heat treatments, although there may be implications on long-term protein utilization. Therefore, the main finding from this work is that further investigation is required to link the effects of heat treatment observed *in vitro* to *in vivo* observations, and to understand the relevance to human health.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

MF designed and conducted the research, analyzed data, and wrote the paper. MPGB wrote the paper. NG conducted the research and wrote the paper. AMM designed and conducted research, analyzed data, and wrote the paper and had primary responsibility for final content. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.643350/full#supplementary-material

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Protein Intake, IGF-1 Concentrations, and Growth in the Second Year of Life in Children Receiving Growing Up Milk – Lite (GUMLi) or Cow's Milk (CM) Intervention

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The relationship of protein intake with insulin-like growth factor 1 (IGF-1) concentrations in well-nourished children during the second year of life is poorly understood. The aim of this study was to explore the effect of a reduced-protein Growing Up Milk Lite (GUMLi) or unfortified cow's milk (CM) on protein intake, growth, and plasma IGF-1 at 2 y. An exploratory analysis of a sub-sample of Auckland-based children ($n = 79$) in the GUMLi trial (a double-blind, randomised control trial, $N = 160$) completed in Auckland and Brisbane (2015–2017) was conducted. One-year old children were randomised to receive a reduced-protein GUMLi (1.7 g protein/100 mL) or a non-fortified CM (3.1 g protein/100 mL) for 12 months. Blood sampling and anthropometric measurements were made at 1 and 2 y. Diet was assessed using a validated food frequency questionnaire. Total protein intake (g/d) from all cow's milk sources was 4.6 g (95% CI: −6.7, −2.4; $p < 0.005$) lower in the GUMLi group after 12 months of the intervention, with a significant group-by-time interaction ($p = 0.005$). Length-for-age (LAZ) and weight-for-length (WLZ) z-scores did not differ between groups, however, mean body fat % (BF%) was 3.2% (95%CI: −6.2, −0.3; $p = 0.032$) lower in the GUMLi group at 2 y. There was no difference between the intervention groups in relation to IGF-1 and IGF-BP3 ($p = 0.894$ and 0.698, respectively), with no group-by-sex interaction. After combining the groups, IGF-1 concentration at 2 y was positively correlated with parameters of growth (all $p < 0.05$), total cow's milk intake ($p = 0.032$) after adjusting for sex, breastfeeding status, and gestation. Randomisation to a reduced protein GUMLi resulted in small reduction in %BF and lower total protein intakes but had no effect on growth. Plasma IGF-1 concentrations were independently associated with total protein intake from cow's milk at

2 y, highlighting a potential area of the diet to target when designing future protein-related nutrition interventions.

Clinical Trial Registration: Australian New Zealand Clinical Trials Registry number: ACTRN12614000918628. Date registered: 27/08/2014.

Keywords: cow's milk, Young Child Formula, growing-up milk, young children, IGF-1, growth

BACKGROUND

During the transition to a family-style diet, a child's protein intake increases significantly, often exceeding physiological demands and creating an imbalance between age-related protein requirements and growth velocity (1, 2). The World Health Organization has suggested lower protein intakes of ≤15% total daily energy intake (EI) as safe levels of protein intake for infants and young children (3). However, recent observational cohort studies of Australian (4), Belgian (5), and Irish children (6) have reported intakes of 2–3 times country-specific recommendations.

The Early Protein Hypothesis, first proposed by Rolland-Cachera et al. (7), states that high protein intake in excess of metabolic requirements, usually associated with formula during early infancy and cow's milk from 1 y of age, has been correlated with increased secretion of growth mediators insulin and insulin-like growth factor I (IGF-1) which enhances fat deposition and weight gain (7–12) alongside risk of obesity and adiposity associated disease (13). It is difficult to ascertain whether early life protein intake sets a precedent for continued high protein intake throughout childhood. However, the association between dietary protein intake exceeding 15% total EI during complementary feeding with increased linear growth, weight gain, and measures of adiposity such as BMI *z*-score during the first 2 y of life and beyond has been reported (9, 14–16).

With Dietary Guidelines recommending the inclusion of cow's milk from 1 y of age (17), milk continues to be a significant determinant of total energy and protein intake during early childhood (4, 5). Several studies have reported an association of %EI from cow's milk with secretion of IGF-1 and measures of adiposity in later childhood (18–23). Of note, a stronger growth-stimulating effect has been reported in children with compromised nutritional status compared to well-nourished children (21, 23, 24). Thus, it appears that protein quantity and quality (including source) combined with nutritional status are factors driving this obesogenic relationship.

The relationship between protein intake and stimulation of IGF-1 is an important consideration in the context of Growing Up Milks (GUM) or Young Child Formula (YCF), which are often a significant protein source in a child's diet (4, 5, 25) and are frequently given to children in higher-income countries (26, 27). The aim of this study was to explore the effect of a reduced-protein Growing Up Milk Lite (GUMLi) or unfortified cow's milk (CM), commencing at 1 y on total protein intake, plasma IGF-1 and IGFBP-3 concentrations and growth at 2 y in a sub-sample of Auckland-based children participating in the GUMLi randomised controlled trial.

Abbreviations: CM, cow's milk; GUM, Growing Up Milk; GUMLi, Growing Up Milk Lite; IGF-1, insulin-like growth factor 1; IGFBP-3, insulin-like growth factor binding protein 3.

METHODS/DESIGN

Study Design

This study represents an exploratory analysis of the GUMLi Trial in a sub-sample of Auckland-based children. The GUMLi trial was a multi-centre double-blind randomised, comparator-controlled trial designed to compare the effect on body composition at 2 y of a reduced protein GUM (GUMLi) fortified with iron, vitamin D, pre- and probiotics (synbiotics) vs. unfortified CM as part of a whole diet for 12 months (28). The trial was multisite, conducted in Auckland, New Zealand and Brisbane, Australia (2015–2017). **Figure 1** shows the study outline. Ethical approvals were obtained from the Northern B Health and Disability Ethics Committee (HDEC) of the New Zealand Ministry of Health (HDEC reference number 14/NTB/152) and the University of Queensland Medical Research Ethics Committee (MREC) in Brisbane, Australia (reference number 2014001318). Written, informed consent to participate in the trial was provided by primary caregivers of all enrolled children.

Participants

Healthy children, 1-year of age (±2 weeks) were recruited from Auckland ($n = 108$) and Brisbane ($n = 52$). Participants were randomised 1:1 to one of two treatment groups: GUMLi or whole pasteurised and homogenised, unfortified cow's milk (CM), both provided in powder form for a period of 12 months. Surplus plasma samples were available for the current analysis in a sub-sample of Auckland children only ($n = 79$) at baseline and month 12 of the intervention. No surplus samples were available from children enrolled in the Brisbane arm of the GUMLi trial.

Study Intervention and Monitoring

The full study design is described in detail elsewhere (28). Briefly, participants were randomly assigned a unique numeric identifier on randomisation into the trial. Data were consolidated using Research Electronic Data Capture Software (REDCap) (29), a secure, password-protected web application, managed by the University of Queensland. The study milks were provided in powder form with instructions on reconstitution with water. The milks differed in the amount of cow's milk protein per 100 mL (22 and 12% of energy, CM and GUMLi, respectively) however, were energy-matched (245 kJ and 249 kJ/100 mL, CM and GUMLi, respectively). GUMLi was lower in total fat

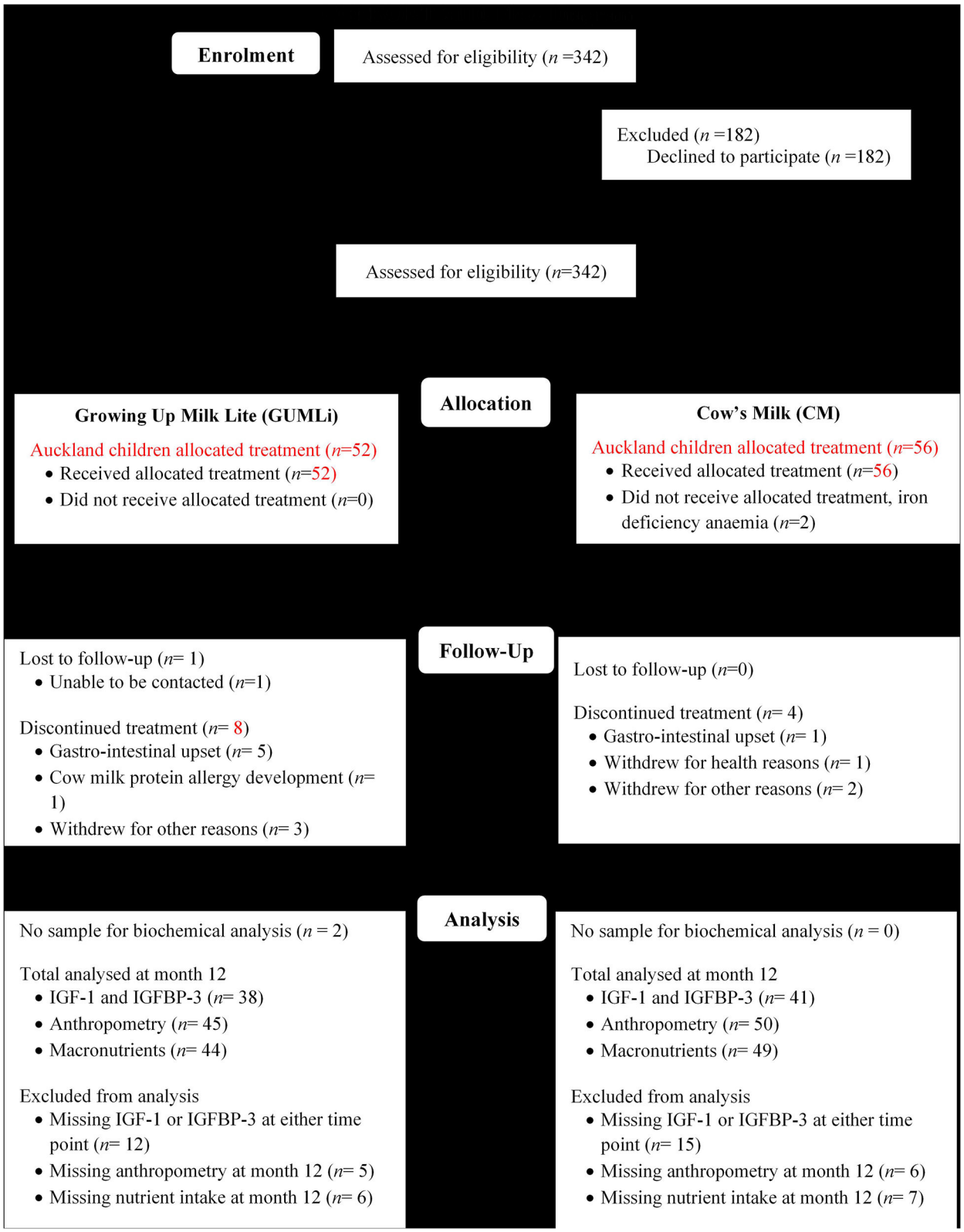

FIGURE 1 | Modified CONSORT flow diagram showing Auckland GUMLi Trial participant randomisation, allocation, follow up and study analysis. CONSORT, CONsolidated Standards Of Reporting Trials; IGF-1, insulin-like growth factor 1; IGFBP-3, insulin-like growth factor binding protein 3.

(supplied as milk fat with added long-chain polyunsaturated fatty acids), was fortified with micronutrients, including iron and vitamin D, and probiotics, and prebiotics (synbiotics). GUMLi had no added sucrose, dextrose, or flavours, however, had higher total carbohydrate content, attributed to higher lactose content. The whey:casein ratio in GUMLi was matched to CM at 80:20 (**Supplementary Table 1**). Participants were requested to consume at least 300 mL of the study milk per day. Adherence was measured at monthly intervals throughout the 12 month intervention and was defined as consumption of ≥300 mL study milk/d on 80% of the days within the previous month. Total adherence to the study protocol was calculated as the mean across the 12 month intervention period. Parents were not provided with any dietary advice during the intervention and continued breastfeeding was encouraged and supported throughout the trial. Participants that were consuming any formula prior to randomisation were asked to discontinue at baseline and only offer the study milk (CM or GUMLi) for the duration of the intervention. If children required additional milk each day beyond the 300 mL of intervention milk, parents were asked to offer whole cow's milk, as per the Dietary Guidelines for children >1 year of age (17).

Dietary Intake

Dietary intake was assessed using the interviewer-administered Eating Assessment in Toddlers Food Frequency Questionnaire (EAT FFQ) at baseline and month 12 of the intervention (30, 31). The EAT FFQ is a previously validated, New Zealand-specific FFQ designed to describe dietary intake of children 1–2 y and has been validated and calibrated in the GUMLi population, following additions of food items and to determine the validity of providing an estimate of vitamin D intake from food (32). Intakes of energy (kcal/d) and protein (g/d) were calculated as previously described, using a custom written programme in MATLAB® (MathWorks Inc., United States) verified by hand calculations (33). Intake of cow's milk as a liquid was hypothesised to influence IGF-1 concentrations, therefore was a variable of interest. Cow's milk was defined as whole and skim milk, served as a drink or with/in food. Both the unfortified CM and intervention GUMLi were cow's milk-based products, therefore counted towards total daily cow's milk intake.

Anthropometric Measurements

Standardised anthropometric measurements including weight (kg), recumbent length (cm), BMI (kg/m^2), and body composition determined by a single frequency bioelectrical impedance (BIA) device (Bodystat 1500 MD) were performed by trained GUMLi study staff and have been described in detail elsewhere (28).

Sample Collection and Analysis

A non-fasted 3 mL finger prick capillary sample was collected by an experienced paediatric phlebotomist at baseline and month 12 of the intervention. Samples were collected into a 0.25 mL EDTA tube (full blood count), two 0.5 mL serum separate tubes (c-reactive protein and iron studies) and a plain 0.5 mL tube for 25-hydroxyvitamin D concentration measurements. Within 2 h of collection, blood in the serum separator tubes was centrifuged for 10 min at 3,000 rpm to separate the serum from whole blood. Samples were analysed by the local pathology laboratory, LabTests Auckland (c-reactive protein and iron studies) as part of the trial secondary analyses (34). LabTests regularly participate in external quality assurance testing and accuracy and precision of all laboratory measurements were determined via analysis of appropriate standards and controls. After biochemical analysis of the trial secondary outcomes (iron and vitamin D status) (34), surplus serum samples were frozen and stored (−80°C) at LabTests, Auckland. These samples were transferred to the Liggins Institute, the University of Auckland, defrosted and concentrations of IGF-1 and IGFBP-3 measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (human serum/plasma IGF-1 and IGFBP-3 ELISA, Mediagnost, Germany). All laboratory steps were performed as per the manufacturer's instructions without any modification. The intra- and inter-assay coefficients of variation were 2.2 and 2.4% for IGF-1, respectively and 3.7 and 11% for IGFBP-3, respectively. Sensitivity was 0.7 ng/mL.

Statistical Methods

Baseline descriptive statistics, including participant and parental characteristics were summarised by treatment group. Categorical variables were described as frequencies and percentages, normally distributed continuous variables as means ± SD, and non-normal continuous data as medians and quartiles. IGF-1 and IGFBP-3 concentrations were not normally distributed and were log transformed and the geometric mean displayed. All statistical analyses were performed using SPSS, version 26 for Windows (SPSS Inc., Chicago, IL, USA). Data was tested for normality using Kolmogoroc-Smirnov and Shapiro-Wilk tests, with $p > 0.05$ for either test treated as normally distributed. All model assumptions were checked and statistical tests were two-sided with a significance level of $p < 0.05$. Baseline variables between the GUMLi and CM groups were compared using independent *t*-tests for parametric variables and Mann-Whitney for non-parametric variables. For categorical variables, chi-squared tests were used, or Fisher's exact test with small cell counts (<5).

Repeated-measures ANOVA were used to evaluate the main effect of time and group and their interactions with the dependent variables. Student's *t*-test was used as a *post-hoc* analysis to compare values between groups at each time point (paired) and over time (independent) and changes over time within each group (paired). ANCOVA regression models were used to investigate the effect of the study milks on anthropometric, dietary and biochemical variables after 12 months of the intervention, adjusting for baseline outcomes, and child sex. A hierarchical multiple linear regression was used to test associations between IGF-1 and variables (growth and nutrient intake), adjusting for confounders. Partial correlations were performed on the sub-sample to identify relationships between growth, biochemical and dietary intake variables, controlling for sex, whether the child was still breastfeeding at 2 y, and gestation due to their known relationships with IGF-1.

RESULTS

Participant Characteristics

A total of 79 (73%) children from the Auckland centre of the GUMLi trial (full Auckland cohort $n = 108$) had biochemical samples available for IGF-1 and IGFBP-3 concentration analysis at both baseline and month 12 of the intervention (**Figure 1**). Baseline characteristics are summarised in **Table 1**. There were no significant group differences for baseline sociodemographic characteristics, apart from gestation, where 11% ($n = 6$) of the CM children were reported as being born <37 weeks gestation compared to 0% in the GUMLi intervention ($p = 0.019$). Three of the children born <37 weeks and randomised to the CM intervention were VLBW ($n = 1$) or low birth-weight (LBW, $n = 2$) and 2 children randomised to the GUMLi intervention were LBW. The remaining child with a gestation of <37 weeks was not considered low birth weight. Forty percent of the participants were receiving breastmilk at baseline. There were no group differences in anthropometric characteristics at baseline, and prior to randomisation total baseline energy and protein intakes did not differ between groups.

TABLE 1 | Birth and baseline characteristics of included Auckland participants ($n = 108$).

Characteristic	Study Group		*p*-value
	CM ($n = 56$)	GUMLi ($n = 52$)	
Sex, *n* (%)			0.190*
Boy	35 (63)	26 (50)	
Girl	21 (37)	26 (50)	
Gestation, *n* (%)			0.015*
Term	50 (89)	52 (100)	
<37 weeks	6 (11)	0 (0)	
Still breastfed at enrolment, *n* (%)			0.325†
Yes	25 (45)	18 (35)	
No	31 (55)	33 (64)	
missing		*1 (2)*	
Attends day care, *n* (%)			0.795*
Yes	32 (57)	31 (60)	
No	24 (43)	21 (40)	
Child anthropometric measurements			
Baseline BMI (kg/m^2), mean ± SD	17.5 ± 1.3	17.3 ± 1.3	0.981§
WAZ, median (Q1,Q3)	0.5 (−0.3, 1.3)	0.6 (0.2, 1.2)	0.742‡
LAZ, median (Q1,Q3)	0.1 (−0.6, 0.9)	0.4 (−0.6, 1.4)	0.190‡
zBMI, mean ± SD	0.6 ± 0.9	0.4 ± 0.8	0.935§
zWeight-for-length, mean ± SD	0.6 ± 0.9	0.5 ± 0.8	0.684§
Body Fat (%), mean ± SD	23.4 ± 6.8	22.4 ± 6.8	0.775§
Nutrient intake			
Energy (kcal/day), mean ± SD	1336.4 ± 532.2	1254.1 ± 380.1	0.680§
Protein (g/day), median (Q1, Q3)	46.0 (36.6, 58.0)	50.3 (38.0, 60.7)	0.412‡
Protein (%EI), median (Q1, Q3)	15.5 (14.1, 17.5)	16.3 (14.5, 18.0)	0.480‡
Protein (g/kg/day), median (Q1, Q3)	4.8 (3.9, 5.9)	4.8 (4.0, 6.0)	0.591‡
Total milk intake‖ (mL/day), median (Q1, Q3)	470.6 (68.8, 719.6)	497.9 (160.3, 645.8)	0.883‡
Serum biomarkers			
IGF-1 (ng/mL), mean¶ ± SD	77.3 ± 58.0	71.0 ± 66.1	0.324‡
IGFBP-3 (ng/mL), mean¶ ± SD	2709.6 ± 759.2	2678.4 ± 991.7	0.500‡

*Chi-squared.
†Fisher's exact test performed due to <5 expected counts.
‡Mann-Whitney for non-parametric variables.
§Student t-test.
‖Includes whole CM, CM based formula, Toddler Milk/Young Child Formula.
¶Geometric mean.
BMI, body mass index; CM, cow's milk; EI, energy intake; GUML, Growing Up Milk – Lite; IGF-1, insulin-like growth factor 1; IGFB-3, IGF binding protein 3; LAZ, length-for-age z-score; Q, quartile; WAZ, weight-for-age z-score; Z z-score.

Dietary Intake

Total energy (kcal/d) and absolute protein intakes (g/d) increased over time, however, they did not differ significantly between groups at baseline or month 12 of the intervention (**Table 2**). There was considerable variation in the mean intake of cow's milk (whole and skim) as a drink in addition to the 300 mL of intervention milks, demonstrated by large standard deviations in volume (mean (SD), CM 101.5 (100.0) mL/d and GUMLi 88.7 (107) mL/d). There were no differences in additional cow's milk intakes (whole and skim cow's milk as a drink) between intervention groups after 12 months of the intervention milks ($p = 0.423$). Energy-adjusted daily protein intake was 2.8 g/1,000 kcal (95% CI: −5.4, −0.1; $p = 0.040$) lower in the GUMLI group after adjusting for baseline protein intake and child sex. Total protein intake (g/d) from all cow's milk drink sources was 4.6 g (95% CI: −6.7, −2.4; $p < 0.005$) lower in the GUMLi group after 12 months of the intervention. Notably, percent total protein intake (%PI) from cow's milk was 6.9% (95%CI: −10.4, −3.4; $p < 0.005$) lower in the GUMLi group at month 12. This resulted in a 4% increase and 30% decrease in %PI from cow's milk at month 12 in the CM and GUMLi groups, respectively.

Anthropometry

Mean weight, length, BMI measurements, their associated *z*-scores and measures of adiposity are summarised in **Table 2**. No significant group-by-time interaction was seen for any of the anthropometric or growth variables (**Supplementary Table 2**). Children randomised to receive GUMLi had a 3.2% (95% CI: −6.2, −0.3; $p = 0.032$) lower body fat percentage (BF%) at 2 y compared to children randomised to receive CM, however, no significant group-by-time interaction was seen.

Serum Biomarkers

Children randomised to receive GUMLi had IGF-1 concentrations 19.6 ng/mL (95% CI:−41.0, 1.9; $p = 0.0.073$) lower than children in the CM group. IGF-1 significantly increased from 1 to 2 years of age ($p = 0.003$) in both groups, however, IGFBP-3 did not change significantly between 1 and 2 years of age and were comparable between groups (**Table 2**). The milk interventions had no effect on IGF-1 or IGFBP-3 when analysed separately by sex (**Supplementary Table 3**).

Correlations

There was no effect of the milk intervention on IGF-1 concentrations at month 12 of the intervention (**Table 2**). Therefore, the CM and GUMLi groups were combined and

TABLE 2 | Anthropometric, macronutrient, and biochemical outcomes at baseline and month 12 of the intervention and associated treatment effects in a subset of Auckland participants ($n = 79$) participating in the GUMLi randomised controlled trial ($N = 160$).

	Intervention Group					Intervention effect	*P*-value*	*P*-value†		
	n	CM		*n*	GUMLi					
		Baseline	Month 12		Baseline	Month 12			Group-by-time	Main effect of time	Main effect of group
Anthropometry	50			45							
Weight											
kg		10.0 ± 1.3	12.9 ± 1.7		9.9 ± 1.1	12.8 ± 1.3	−0.1 (−0.5, 0.2)	0.394	0.578	<0.005	0.920
z score‡		0.5 ± 1.1	0.6 ± 1.0		0.5 ± 0.9	0.6 ± 0.9	−0.1 (−0.3, 0.1)	0.467	0.417	0.705	0.811
Length											
cm		75.3 ± 2.9	88.3 ± 3.8		75.7 ± 3.1	88.7 ± 3.6	0.1 (−0.8, 0.7)	0.842	0.982	<0.005	0.253
z score‡		0.1 ± 1.1	0.4 ± 1.2		0.3 ± 1.1	0.6 ± 1.1	0.0 (−0.3, 0.2)	0.953	0.761	0.001	0.244
BMI											
kg/m^2		17.5 ± 1.4	16.5 ± 1.5		17.3 ± 1.1	16.2 ± 1.2	−0.2 (−0.6, 0.3)	0.427	0.612	<0.005	0.359
z score‡		0.6 ± 0.9	0.5 ± 1.0		0.4 ± 0.8	0.2 ± 0.9	−0.1 (−0.4, 0.2)	0.340	0.460	0.011	0.356
WAZ‡		−0.12 ± 1.2	0.25 ± 1.1		−0.07 ± 0.9	0.24 ± 1.0	−0.0 (−0.3, 0.2)	0.773	0.720	<0.005	0.772
WLZ‡		0.6 ± 0.9	0.5 ± 1.0		0.5 ± 0.8	0.3 ± 0.8	−0.1 (−0.4, 0.1)	0.309	0.377	0.008	0.519
LAZ‡		0.14 ± 0.9	0.40 ± 1.1		0.34 ± 1.0	0.54 ± 1.0	0.0 (−0.3, 0.2)	0.713	0.595	0.010	0.284
Body Fat											
%		24.4 ± 6.3	23.5 ± 6.8		22.9 ± 6.7	19.6 ± 6.9	−3.2 (−6.2, −0.3)	0.032	0.180	0.071	0.039
Free Fat Mass											
kg		7.6 ± 1.2	9.9 ± 1.4		7.6 ± 1.1	10.2 ± 1.5	0.2 (−0.2, 0.7)	0.291	0.279	<0.005	0.278
Fat Mass											
kg		2.4 ± 0.7	3.1 ± 1.1		2.3 ± 0.7	2.5 ± 0.9	−0.5 (−0.9, −0.1)	0.028	0.082	0.028	0.039
Fat Mass Index											
kg/m^2		4.3 ± 1.2	3.9 ± 1.4		4.0 ± 1.2	3.2 ± 1.2	−0.7 (−1.2, −0.1)	0.020	0.170	0.005	0.028
Macronutrients	50			45							
Energy											
kcal/d		1336.4 ± 532.2	1539.2 ± 406.7		1254.1 ± 380.1	1637.1 ± 527.1	152.1 (−25.8, 330.1)	0.093	0.086	<0.005	0.605
From CM (kcal/d)		312.6 ± 2061	275.3 ± 97.3		294.1 ± 195.4	257.6 ± 106.5	−12.7 (−54.1, 28.7)	0.544	0.999	0.179	0.579
Protein											
g/d		51.3 ± 18.3	74.2 ± 21.8		50.5 ± 19.4	74.8 ± 27.2	2.3 (−7.5, 12.0)	0.646	0.661	<0.005	0.788
From CM (g/d)		10.3 ± 6.5	15.0 ± 5.6		10.3 ± 7.0	10.3 ± 4.8	−4.6 (−6.7, −2.4)	<0.005	0.005	0.034	0.024
g/1,000 kcal		39.0 ± 7.9	47.3 ± 6.9		39.2 ± 7.1	44.6 ± 6.2	−2.8 (−5.4, −0.1)	0.040	0.146	<0.005	0.197
g/kg/d^2		5.1 ± 1.7	5.8 ± 1.7		5.1 ± 1.9	6.0 ± 2.4	0.3 (−0.6, 1.1)	0.543	0.524	0.003	0.803
%PI from CM		20.9 ± 13.8	21.7 ± 9.1		21.1 ± 14.6	14.8 ± 7.9	−6.9 (−10.4, −3.4)	<0.005	0.024	0.131	0.086
%EI from CM		3.2 ± 2.0	4.1 ± 1.6		3.1 ± 2.3	2.7 ± 1.4	−1.5 (−2.1, −0.9)	<0.005	0.002	0.797	0.034
Biochemical	41			38							
IGF-1§											
ng/mL		77.3§ ± 58.0	105.5§ ± 65.0		71.0§ ± 66.1	95.7§ ± 43.2	−19.6 (−41.0, 1.9)	0.073	0.698	0.003	0.126
IGFBP-3§											
ng/mL		2709.6§ ± 759.2	2911.4§ ± 821.0		2678.4§ ± 991.7	2910.4§ ± 714.0	−134.8 (−446.0, 176.4)	0.391	0.874	0.479	0.518

All data are mean ± SD.

**An ANCOVA model was used to test the difference between the two groups, adjusting for baseline outcome, child sex and multiple comparisons (Bonferroni).*

† Repeated-measures ANOVA for group (GUMLi compared with CM), with interaction between treatment and time, adjusting for child sex, $P < 0.05$.

‡ The z-score is calculated using World Health Organization child growth standards.

§ Geometric mean.

CM, Cow's Milk; EI, Energy Intake; GUMLi, Growing Up Milk Lite; IGF-1, insulin-like growth factor I; IGFBP-3, insulin-like growth factor binding protein 3; LAZ, length-for-age z-score; PI, Protein Intake; WAZ, weight-for-age z-score; WLZ, weight-for-length z-score.

associations between IGF-1, IGFBP-3, anthropometry and diet were investigated at baseline and month 12 of the intervention, adjusting for sex (**Supplementary Tables 4, 5**). At baseline, IGF-1 concentrations were positively correlated with size (zWFL, zBMI, WAZ; all $p < 0.01$, and LAZ, $p = 0.020$). There was a significant positive correlation between IGF-1 and protein per gramme of body weight ($p = 0.020$) and %EI from cow's milk protein ($p = 0.044$). After 12 months of the intervention, IGF-1 at 2 y

TABLE 3 | Standardised beta coefficients from multiple regression predictors of IGF-1 at 2 years of age in a subset of Auckland participants ($n = 79$) participating in the GUMLi randomised controlled trial ($N = 160$).

	Sex	Length-for-age *z*-score	Body fat percent at age 2 years	Total protein intake (g/d) from CM at age 2 years
Base model	0.327†	0.209		
Model 1	0.325†	0.201	−0.031	
Model 2	0.355†	0.159	−0.063	0.247*

CM, Cow's Milk; IGF-1, insulin-like growth factor I; IGFBP-3, insulin-like growth factor binding protein 3; PI, Protein Intake.
$^{*}p < 0.05$; $^{\dagger}p < 0.01$.
Base model: child sex, length-for-age at age 2 years.
Model 1: child sex, length-for-age at age 2 years, body fat percent at age 2 years.
Model 2: child sex, length-for-age at age 2 years, body fat percent at age 2 years, total protein intake from CM.

remained positively correlated with size (zWF, zBMI, and LAZ $p < 0.05$, WAZ $p < 0.006$) after adjustment for sex, breastfeeding status, and gestation. Change in IGF-1 from 12 to 24-months-of-age was significantly positively correlated with weight (zWFL, and zBMI $p < 0.05$ and WAZ, $p < 0.01$). There was a significant association between IGF-1 and total cow's milk intake ($r_s = 0.280$, $p = 0.032$) and a trend towards an association between %EI from cow's milk protein ($r_s = 0.245$, $p = 0.061$) adjusted for sex, breastfeeding status, and gestation (**Supplementary Table 5**).

Further, to establish whether associations between IGF-1 at 2 y and protein intake from cow's milk were independent of confounding, a hierarchical multiple linear regression was performed incorporating sex and LAZ at 2 y in the base model (**Table 3**). LAZ was included in the base model due to its positive bivariate correlation with IGF-1 at 2 y. Body fat percent was not significantly associated with IGF-1 when added into the model (Model 1). In Model 2 we added total protein intake from cow's milk at 2 y and all statistically significant relationships from Model 1 remained, with a significant association between total protein intake from cow's milk and IGF-1. In addition, total protein intake (g/d) from cow's milk at 2 y was positively associated with IGF-1 at 2 y ($\beta = 0.25$, 95%CI 0.001, 0.018; $p = 0.03$).

DISCUSSION

In this sub-set of well-nourished, healthy, Auckland-based children from the GUMLi randomised control trial cohort we found no effect of the milk interventions on IGF-1 concentrations at 2 y across the two groups or by sex. As expected, adjusted protein intake (g/1000 kcal), total protein intake from cow's milk-based drinks, and %EI from cow's milk protein decreased significantly in the GUMLi group over the 12-month duration of the trial, with significant group-by-time interactions evident at 2 y. There were no independent effects of the milk intervention at 2 y for anthropometric variables, except BF% and FFM.

Our findings that a higher protein intake from cow's milk in early life is associated with a small increase in adiposity is consistent with observational cohorts (16, 24, 35, 36) and previous randomised controlled trials by Weber et al. (10) and Koletzko et al. (37), which demonstrated a positive causal effect of high protein intakes during infancy on BMI and weight gain in early life. *Post-hoc* analysis revealed a significant decrease in WLZ over the 12-month duration of the study in the GUMLi group only, suggestive of a decreased risk of overweight in the GUMLi group, further supported by the small but significant difference in BF% between groups alongside significant linear growth. These growth patterns are similar to those observed in children randomised into a dairy vs. a meat-based complementary diet from 5 to 12 months of age reported by Tang et al. (20) in their follow up of infants at 2 y. As our study evaluated the effect of two dairy-based protein interventions direct comparisons with meat vs. milk studies is not possible. However, provides some evidence of the potential for a combined effect of the protein-source (even within common animal sources of protein i.e., dairy vs. meat) and the complete food matrix on growth, rather than total protein intake (20, 21, 38). This warrants further research in early childhood.

Globally, current protein intakes in 12–36 months old children are high (39), with a greater risk for increased weight gain, body fat deposition and later risk of obesity, adiposity, and associated comorbidities induced by animal proteins (predominantly dairy protein in the form of cow's milk) compared to plant-based proteins (14). The relationship between protein intake and stimulation of IGF-1 is an important consideration in the context of Growing Up Milks (GUM or YCF), which are frequently given to children in higher-income countries and remains a significant source of energy and protein, with intakes ≤500 mL/d providing up to 50% of total protein requirements from 1 y (6, 15, 40, 41). The GUMLi trial study design is unique in that the intervention duration was throughout the second year of life, providing an opportunity to explore the role of diet, particularly the influence of cow's milk as a drink on IGF-1 concentrations and growth beyond 1 y. We hypothesised that the children randomised to receive GUMLi would have lower IGF-1 concentrations, driven by the 45% difference in grammes of protein per 100 mL of the intervention milks. Whilst this analysis contributes to the evidence base exploring the relationship between cow's milk, growth, and IGF-1, it does not afford the ability to infer causality or determine the increased risk associated with exceeding current protein recommendations in the second year of life due to insufficient numbers in each group to detect clinically meaningful differences in outcomes.

Energy-adjusted protein intakes in both groups were higher than previously reported in cross-sectional data of children under 2 y from the US (20, 42), Belgium (5), Ireland (6), and New Zealand (43). This is possibly due to the higher total cow's milk intake in the study (453 mL/d and 431 mL/d, CM and GUMLi groups, respectively) compared to previously reported studies. Differences in the contribution of cow's milk to total protein intake were apparent, with children randomised to the CM group having a 6.9% greater protein intake from cow's milk as a percentage of total daily protein intake compared to the GUMLi group. This difference would equate to a 4.6 g difference in total protein intake from CM, or an additional 150 mL CM per day.

Despite the milk interventions having no independent effects on IGF-1 concentrations, total CM intake (mL/d) at 2 y was independently associated with IGF-1 concentrations at 2 y. The cross-sectional relationship between energy-adjusted protein intake and IGF-1 concentrations in healthy, well-nourished children at 2 y is similar to that reported by Larnkjaer et al. (22), where IGF-1 concentrations were associated with protein intake reported as a percentage of total energy intake at 1 y of age. Providing evidence that total protein intake, particularly protein intake from CM, increases IGF-1 concentrations relative to a child's total energy intake (23, 24, 44). This represents an area of research that warrants further investigation (45).

Limitations

A limitation of this analysis, is that it is an exploratory analysis of the GUMLi randomised controlled trial which was originally powered to detect a 0.5 SD of difference in body fat percent at 2 y and not differences in IGF-1 concentrations or other anthropometric measurements. Excess frozen serum samples were only available for a subset of the Auckland cohort and not the Brisbane cohort. However, the Auckland cohort encompassed 68% of the GUMLi trial sample. Serum urea nitrogen (SUN), an indicator of total protein intake was not measured due to limited serum sample volume and would have strengthened this analysis as increases in SUN have been reported in 9 and 12-month-old infants randomised to a whole milk intervention, with a trend for difference between milk intervention groups of whole milk and formula (22). Absolute quantities of additional protein sources e.g., from partial breastfeeding were not included in the analyses as this could not be quantified. Both intervention milks delivered the same whey:casein ratio of cow's milk protein, but differed in their delivery of fat (including long chain polyunsaturated fatty acids) and synbiotics. The impact of these compositional differences on fasting insulin was not measured and this should be considered when interpreting these results. Whilst there were no differences between groups in IGF-1 concentrations at 2 y, the >60% difference in total carbohydrates between the CM and GUMLi interventions must be considered, as higher levels of simple carbohydrates such as lactose will have an increased effect on insulin concentrations (44).

CONCLUSION

Whilst the reduced protein GUMLi milk intervention in comparison to CM resulted in a small but significant effect on percentage body fat, it had no effect on IGF-1 concentrations at 2 y. Protein intake from CM was positively associated with IGF-1 concentrations on cross-sectional analysis at 2 y. CM remains an important source of nutrition in young children, providing nutrients outside of protein, however, further consideration as to how much it contributes to total protein intakes in early childhood is warranted. The second year of life should be considered as a specific time point where protein-specific dietary interventions could be targeted as children transition to the family diet in an effort to reduce the risk of overweight and adiposity in later childhood.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors upon request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved the Northern B Health and Disability Ethics Committee (HDEC) of the New Zealand Ministry of Health (HDEC reference number 14/NTB/152) and the University of Queensland Medical Research Ethics Committee (MREC) in Brisbane, Australia (reference number 2014001318). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

CW, PD, and CG: developed the GUMLi Trial. AL, MM, TM, and RH: conducted the study data. AL and CW: wrote the manuscript. AL: conducted the statistical analyses of the data. All authors have read, contributed, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.666228/full#supplementary-material

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Conflict of Interest: AL has received honoraria for presentations and consultations from Danone Nutricia. CW has received honoraria for presentations and consultations from Danone, Nutricia, Pfizer, and Fonterra. CG has received honoraria for consultations from Fonterra. PD has received honoraria for presentations and consultation from Danone Nutricia, Nestle, Bayer, Diary Australia, H+H, Bellamys, Sanulac, and Aspen Nutritionals. RH is currently employed by Reckitt Benckiser Group, however was employed at the University of Queensland at the time of the GUMLi Trial.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Bovine Colostrum and Its Potential for Human Health and Nutrition

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Colostrum is the first milk produced post-partum by mammals and is compositionally distinct from mature milk. Bovine colostrum has a long history of consumption by humans, and there have been a number of studies investigating its potential for applications in human nutrition and health. Extensive characterization of the constituent fractions has identified a wealth of potentially bioactive molecules, their potential for shaping neonatal development, and the potential for their application beyond the neonatal period. Proteins, fats, glycans, minerals, and vitamins are abundant in colostrum, and advances in dairy processing technologies have enabled the advancement of bovine colostrum from relative limitations of a fresh and unprocessed food to a variety of potential applications. In these forms, clinical studies have examined bovine colostrum as having the substantial potential to improve human health. This review discusses the macro-and micronutrient composition of colostrum as well as describing well-characterized bioactives found in bovine colostrum and their potential for human health. Current gaps in knowledge are also identified and future directions are considered in order to elevate the potential for bovine colostrum as a component of a healthy diet for a variety of relevant human populations.

Keywords: bovine colostrum, human health, bioactive proteins, oligosaccharides, infants

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INTRODUCTION

Colostrum is the earliest milk produced from the mammary glands for the first few days after giving birth and is unique in its composition of essential nutrients, immune factors, and oligosaccharides that benefit the newborn (1, 2). In the case of cows, bovine colostrum is produced immediately after calving and quickly wanes to mature milk (3), which lacks the high level of beneficial nutrients found in bovine colostrum. There are several factors affecting the composition and physical properties of colostrum such as individuality, breed, parity, pre-partum nutrition, length of the dry period of cows, and time post-partum (4). Generally, colostrum has more fat, protein, peptides, non-protein nitrogen, ash, vitamins and minerals, hormones, growth factors, cytokines, nucleotides, and less lactose compared to mature milk content. The concentration of these compounds decreases rapidly in the first 3 days of lactation with the exception of lactose content (5–7).

While the consumption of human colostrum by infants has long been recognized as a source of critical bioactive proteins for infants (8), the consumption of animal colostrum is also practiced in many locations beyond the neonatal period (9, 10). In these cultures and regions, colostrum has long been consumed as a health food or for medicinal purposes, with cultural practices centered on the belief that animal colostrum was an important component of the development of healthy children and supportive of healthy or infirmed adults (9, 11, 12). While these cultural or regional beliefs are associated with this practice, the abundance of well-characterized bioactive compounds and selective prebiotic components of this food may further support this cultural knowledge from a scientific perspective.

Historically, liquid fresh colostrum was primarily consumed, but pasteurized colostrum is also commercially available as a standalone drink, though production remains small (13). In European cultures and elsewhere such as India, and Scandinavia, colostrum is also used in the production of cheeses and other traditional foods (14). More recently, dried colostrum is collected and processed as a dietary supplement, which is widely consumed for perceived health benefits (10). In the US and EU, colostrum supplements are marketed for a variety of health benefits, including boosting immunity and gastrointestinal (GI) health. While attractive in concept, there are limitations to this use of dried colostrum, which are typically in a pill or tablet form, given the limited amount of colostrum consumed relative to clinically studied consumption rates.

Still, colostrum is a complex biological fluid and contains significant components which are natural anti-microbial factors for stimulating the maturation of calf immunity (15). In addition, the development and function of the GI tract are shaped by colostrum intake (5, 6, 16–18), and it also affects the metabolic and endocrine systems as well as the nutritional state of neonatal calves (5, 6, 17). Colostrum has muscular-skeletal repair and growth potential in addition to its immune support function and many benefits to health because of its content of bioactive proteins (19). Further, some evidence suggests that the cytokines, immunoglobulins, growth factors, antimicrobial compounds, and maternal immune cells are transferred to the newborn with the feeding of colostrum to support neonatal immunity (20–22). Bovine colostrum has even been purported to treat viral and bacterial infections as a nutraceutical (23). Together, the existing evidence in support of colostrum suggests that there is potential for colostrum to have a significant role in supporting human health as well. While there are other studies which have begun to look at colostrum from other animals (24–27), this review explores the current knowledge on the bovine colostrum in the context of nutrition, its bioactive components, and its potential for human health and nutrition.

Bovine Colostrum Composition

Milk composition changes dramatically over the course of lactation and bovine colostrum is compositionally and nutritionally distinct from mature milk (28). In contrast to mature milk, colostrum has a much higher protein and moderately higher fat content, with substantially less lactose (**Table 1**). This reflects the needs of the developing calf, where the passive transfer of immunoglobulins is critical for health (41).

Further, as the volume of milk production increases over lactation, there is a concomitant decrease in the mineral content of milk (**Table 1**). Thus, colostrum represents a relatively high-protein and lower-carbohydrate solution that can be processed similarly to mature milk in order to reduce fat content and shape the caloric density for desired nutritional applications. Further, milk proteins are considered a "complete protein" source owing to their amino acid profile, and high protein digestibility, especially of whey proteins (42), though colostrum contains higher concentrations of immunoglobulins which are less digestible (**Table 1**).

While current dietary recommendations of protein intake for a healthy adult with minimal physical activity are 0.8 g per kg per day (43, 44), a growing body of evidence suggests that optimal intake may be higher [1.2–1.6 g per kg per day; (45–47)] and this intake should be balanced across meals to promote skeletal muscle protein synthesis (48). Especially in elderly populations, optimal protein intake to reduce skeletal muscle loss associated with aging is often not achieved, which is further compounded by diminished proteolytic activity associated with aging (49). Thus, colostrum may offer an attractive digestible, complete protein source that can be integrated into a calorically-appropriate diet. In addition to macronutrients, bovine colostrum includes vitamins, minerals, and a broad assortment of protein-derived bioactives which may offer additive benefits to its macronutrient profile.

Main Factors Affecting Colostrum Composition

The composition and quality of the bovine colostrum are highly variable due to genetic and environmental factors including individuality, breed, parity, the timing of milking, diseases, pre-partum nutrition, season, length of the dry period of cows, and time post-partum (50–52).

Individual Variation Among Animals

Bovine colostrum quality is different among individuals and between genetic backgrounds (31). For instance, the concentration of immunoglobulin G (IgG) in bovine colostrum and the volume of first milking vary among individual cattle (53, 54). The Jersey cows produce the highest (66.5 g/L) whereas Friesian-Holsteins produce the lowest (41.2 g/L) concentrations of IgG among breeds studied (55). In the case of cow parity, first-calf heifers produce a lower yield of colostrum and lower IgG concentration in colostrum than those cows in their second or greater lactation. The quality of bovine colostrum increases with parity after the second calving, and older cows generally produce the best quality colostrum (54).

Another individual factor is the disease which influences bovine colostrum quality. For instance, mastitis is an inflammation of the mammary gland of the bovine that has negative consequences including low quality of the colostrum. The volume and concentration of bovine colostrum IgG are lower in cows with infected mammary glands than cows with uninfected glands (56). The age of cows also affects the quality of colostrum. Some studies' data are in general agreement that

TABLE 1 | Bovine colostrum and mature milk composition.

Colostrum component	n^a	Mean	Minimum	Maximum	SE	Mature milk
Bovine colostrum						
Fat mg/mL	1,226 (29)	64.00	41.00	83.00	33.20	39.00 (28)
	54 (30)	67.00	20.00	265.00	41.60	
Protein mg/mL	1,226 (29)	140.00	116.00	166.00	36.70	36.00 (28)
	55 (30)	149.20	71.00	226.00	33.20	
Casein mg/mL	– (31)	43.00	–	–	–	25.00 (31)
Whey mg/mL	– (31)	120.00	–	–	–	5.10 (31)
Lactose mg/mL	1,226 (29)	27.00	23.00	31.00	5.50	49.00 (28)
	55 (30)	24.90	12.00	52.00	6.50	
Dry matter mg/mL	55 (30)	276.40	183.00	433.00	58.40	125.00 (28)
Ash mg/mL	55 (30)	0.50	0.20	0.70	0.10	7.00 (28)
IgG mg/mL	1,239 (29)	55.00	38.10	67.80	25.75	0.257 (32)
IgA mg/mL	55 (30)	1.66	0.50	4.40	0.50	0.04–0.06 (12, 30, 33, 34)
IgM mg/mL	55 (30)	4.32	1.10	21.00	1.10	0.03–0.06 (12, 30, 33, 35)
Oligosaccharides mg/mL	– (36)	–	0.70	1.20	–	0.3–0.5 (36)
Lactoferrin mg/mL	55 (37)	0.82	0.10	2.20	0.10	0.10–0.30 (37)
Lactoperoxidase mg/mL	– (38)	–	11.00	45.00	–	13–30 (38)
Ca mg/kg	55 (30)	4,716.10	1,898.00	1,775.10	8,593.50	1,220.00 (39)
	– (40)	1,518.60	–	–	–	
P mg/kg	55 (30)	4,452.10	1,706.29	1,792.40	8,593.5	1,520.00 (39)
	– (40)	1586.00	–	–	–	
Mg mg/kg	55 (30)	733.24	286.07	230.30	1,399.60	120.00 (39)
	– (40)	219.70	–	–	–	
Na mg/kg	55 (30)	1,058.93	526.02	329.70	2,967.80	580.00 (39)
	– (40)	516.70	–	–	–	
K mg/kg	55 (30)	2,845.89	1,159.89	983.20	5,511.40	1,520.00 (39)
	– (40)	1,297.50	–	–	–	
Zn mg/kg	55 (30)	38.10	15.90	11.20	83.60	5.30 (39)
	– (40)	151.00	–	–	–	
Fe mg/kg	55 (30)	5.33	3.09	1.70	17.50	0.80 (39)
		34.66	–	–	–	
Mn[b] mg/kg	23 (30)	0.10	0.11	0.00	0.36	0.20 (39)
	– (40)	2.62	–	–	–	
Vitamin A mg/kg	55 (30)	4.90	1.82	1.40	19.30	460.00 (39)
Vitamin E mg/kg of fat	55 (30)	77.17	33.52	24.20	177.90	2.10 (39)
Vitamin B12 μg/mL	5 (30)	0.60	0.35	0.20	1.10	4.50 (39)

[a] *Number of colostrum samples reported in the referenced study.*
[b] *Part of the samples were quantified as <0.05 and therefore not included in averages.*

older cows have a higher quality of colostrum than younger cows (53, 54, 57). The association between older age and good quality of colostrum is thought to be a result of increased pathogen exposure, improved immunity, and body condition score (31).

Environmental Factors

The timing of the bovine colostrum milking after parturition has significant effects on concentrations of IgG in the bovine colostrum. Early or immediate colostrum milking will significantly increase colostrum quality. Moore et al. (58) reported that colostrum collected 6, 10, and 14 h after parturition has lower IgG concentration than colostrum collected 2 h after parturition. Another study also showed that bovine colostrum quality is highest immediately after parturition of North American herds, but it decreased when milking was delayed (53, 58). Bovine colostrum quality is also affected by the calving season. Cows calving during the summer months have lower quality colostrum than those calving in the autumn months (53). The bovine colostrum fat percentage is at 24 and 48 h after birth is affected by the calving season. Animals born in autumn-winter seasons have a higher colostrum fat percentage than those in calving in spring-summer seasons. One cause may be differences in metabolism, feed, and water consumption in different seasons (59, 60).

The dry period length is an important period for cows which lasts ∼6–8 weeks. This period is needed for the renewal of

milk secretion tissue, preparation for lactation, and completion of fetus development (61–63). Colostrum starts to be secreted in the last 15–20 days of the dry period and its composition changes continued until parturition (62, 64). Le Cozler et al. (65) also reported that there is a positive coefficient of correlation ($R^2 = 0.22$; $P < 0.01$) between IgG concentration and dry period length (65).

Fats

Colostrum contains a higher percentage of fat than mature milk (66) and the composition of these fats is also distinct. O'Callaghan et al. (67) examined the composition of colostrum and the changes observed during the transition to mature milk, reporting that colostrum is higher in palmitic, palmitoleic, and myristic acids, relative to mature milk (67). While these fat profiles are well-suited to the developing calf (68), the profiles of these fats and the higher concentration of saturated fat have been associated with long-term negative health outcomes, though there is some disagreement within the literature as to the level of support for the role of dairy fats in cardiovascular disease (69). There is evidence that these fatty acids play a role as signaling molecules and, as dietary fatty acids, contribute to the regulation of lipogenesis in the liver (70). Further, many vitamins found in milk are fat soluble (e.g., vitamin A, D) and removal of these fats also reduces the concentration of these vitamins in colostrum.

It is of relevance to consumers that the advances in dairy technology which enable efficient separation of fats from the aqueous fraction of milk (that is, the fraction which contains proteins, carbohydrates, minerals, and some vitamins) enable the reduction or removal of these fats from colostrum, ahead of downstream processing, making the potential for a low-fat or fat-free colostrum product possible. However, some have speculated that the tradeoff between dairy fats and the removal of bioactive found in the fat fraction of dairy foods may not always be a net benefit (69). To resolve this conflict in the literature, it is clear that well-controlled clinical studies investigating the relationship between the dietary fats found in colostrum and health are needed.

Vitamins/Minerals Found in Colostrum

Bovine colostrum contains also high levels of fat-soluble and water-soluble vitamins that are critical to human health (4). Notably, vitamin A is reported to be found at high concentrations in colostrum in a variety of forms including retinol, retinal, retinoic acid, retinyl esters and as provitamin A carotenoids (71–73). Vitamin E, in the form of tocopherols and tocotrienols ($\sim$ mean 77.17 mg/kg) are found in low density lipoproteins in colostrum (4, 30). Vitamin K is also found in greater concentration in colostrum compared to mature milk in two forms, phylloquinone, and menaquinones (71). Vitamin D is found in higher concentrations in colostrum than mature milk (74). Vitamin D has important roles in immune activities and promotes the uptake of calcium and phosphorus in the small intestine (75). It has two major forms as cholecalciferol (vitamin D3) and ergocalciferol (vitamin D2) and their concentration decreases from 1.2 to 0.36 IU. g−1 during the first 5 days post-partum (76). Vitamin C and the B vitamins are also found in the water-soluble fraction of colostrum at a higher concentration compared to mature milk (77) and together provide a natural source of essential vitamins critical to human health.

Bovine colostrum and mature milk are known to be good sources of several minerals especially calcium and phosphorus (75). Recent studies revealed that the mean concentrations of several important minerals in colostrum are significantly higher than in mature bovine milk. Calcium is necessary for the maintenance of calf development and their healthy bones and teeth. Phosphorus is also crucial for the metabolic rate and physiological functions including development of skeletal tissue, energy utilization, protein synthesis, and transport of fatty acids (78). Magnesium is present in a relatively large amount, along with zinc and selenium in bovine colostrum (75).

Bioactive Proteins

Immunoglobulins (Igs)

Immunoglobulins (Igs) are complex proteins, known as antibodies, that make up a significant part of the total protein in bovine colostrum. The immunoglobulins in bovine colostrum mainly come in 3 different varieties called isotypes including IgG (IgG1 and IgG2), IgA, IgM. IgG is the dominant immunoglobulin in bovine colostrum, which makes up 85–90% of the total immunoglobulin content. IgG1 represents 80–90% of the total IgG content in bovine colostrum, followed by IgM, IgA, and IgG2 (23, 79, 80). These immunoglobulins are essential in the survival of the calves and their immune systems and they neutralize enteric pathogens such as bacteria, microbes, and viruses. Using bovine colostrum as a source of antibody preparations to support bovine and human health is an important research subject that has been studied for decades (81).

One of the key differences between mature milk and colostrum is the high concentration of IgG found in colostrum, which reaches up to 50–100 mg/mL in the first days after birth (33, 82, 83). Bovine serum IgG1 and IgG2 concentration decrease before parturition, they are transferred from the blood into the colostrum. In fact, nearly all IgG in colostrum is transferred from bovine serum into the colostrum and milk (84, 85).

The high concentration of IgG is necessary for the survival of calves, which is strongly dependent on the transfer of IgG from bovine colostrum to calves to provide passive immunity as cows cannot transfer IgG through the placenta (86). Indeed, if calves do not receive colostrum immediately after birth, they are prone to infection and will suffer from a higher risk of morbidity and mortality (31, 87, 88).

Lactoferrin

Lactoferrin is a cationic, iron-binding glycoprotein present as about 0.80 mg/mL in bovine colostrum (37). It has multiple functions including antibacterial, antifungal, antiviral, antiparasitic, antitumor and immunomodulatory (anti-inflammatory) effects (23, 35, 89, 90), and is the major protein in the milk serum of all mammals (91). Bovine colostrum-derived lactoferrin has antimicrobial activity by inhibiting the growth of disease-causing protozoa, yeasts, bacteria, and viruses, and lactoferrin may prevent the attachment of pathogens to epithelial cells and help maintain intestinal

permeability and stability (83, 92, 93). Moreover, there are some studies showing that bovine colostrum-derived lactoferrin can increase the proliferation of cells involved in the bone formation such as osteoblasts, and the release of some growth factors from osteoblasts (94, 95).

Furthermore, it is known to play a role in iron uptake in the intestine and activation of phagocytes and immune responses. Receptors for lactoferrin are expressed on intestinal tissue, monocytes, macrophages, neutrophils, lymphocytes, platelets, and on some bacteria (96). Bovine lactoferrin supplements are thought to support the immune system and influence immune cell activity potentially via these antioxidant, antibacterial, and antiviral properties (97). The greatest concentration of this protein is found in colostrum, which has been determined to be four times greater than mature milk (98).

Lactoperoxidase

Lactoperoxidase is a major antibacterial enzyme found in bovine colostrum, it is a basic glycoprotein that catalyzes the oxidation of thiocyanate and generates intermediate compounds with antimicrobial activities (99). The concentration of lactoperoxidase is 11–45 mg/L in bovine colostrum and 13–30 mg/L in mature bovine milk (38). Its concentration in bovine colostrum is low initially, but it reaches the maximum level within 3–5 days after parturition. Lactoperoxidase catalase activity is also higher in bovine colostrum than in mature milk (100, 101).

Lactoperoxidase activity produces toxic oxidation products that inhibit bacterial metabolism by oxidation of essential sulfhydryl groups in proteins. This system is toxic to some gram-positive and negative bacteria like *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Listeria monocytogenes*, *Streptococcus mutans*, and *Staphylococcus aureus* (102). The lactoperoxidase system also inactivates the poliovirus, vaccinia virus, and HIV (93, 103, 104).

Oligosaccharides

Bovine colostrum is a rich source of complex and highly selective oligosaccharides and glycans. The concentration of oligosaccharides in colostrum is 0.7–1.2 mg/mL and the majority of these structures are acidic oligosaccharides which are lower in mature bovine milk (36, 105). Forty distinct oligosaccharides compositions have been detected in bovine colostrum so far (106–108). The total colostrum oligosaccharides differ between cows because of their genetic variability (109). Predominant oligosaccharides in bovine colostrum are 3′ sialyllactose (3′SL), 6′ sialyllactose (6′SL), 6′ siayllactosamine (6′SLN) and disialyllactose (DSL). 3'SL is 70% of total oligosaccharide content in bovine colostrum (105, 107, 110, 111). 3′SL, 6′SL, and 6′SLN levels in colostrum were highest following parturition and decreased by 48 h post-partum, while neutral oligosaccharide level increased (105). Breed specific differences have also been identified in oligosaccharide content. Concentrations of 3′SL, 6′SL, 6′SLN and DSL were found as 867, 136, 220, and 283 μg/mL, respectively, in colostrum from Jersey cows, while these concentrations were 681, 243, 239, and 201 μg/mL, respectively, in Holstein colostrum after parturition (112). Both free oligosaccharides (bovine milk oligosaccharides, BMOs) and complex, conjugated *N*-glycans represent the majority of the prebiotic components of bovine colostrum (113).

While there are many distinctions between BMOs and human milk oligosaccharides (HMOs), there has been significant interest in utilizing milk and colostrum as a source of BMOs for human nutrition and health to modulate the GI microbiome (114). In contrast to HMOs, BMOs are predominantly sialylated (i.e., acidic) oligosaccharides, with a low propensity for fucosylation (106) and a lower structural diversity (106). Recent advancements in enzymatic glycosylation have provided opportunities for the structural enhancement of BMOs to alter their structure to resemble HMOs (115). Several complexities in milk processing have thus far limited the ability of BMOs to be separated from lactose found at high concentrations in milk (114), though solutions have begun to emerge (116) which complicates their utility for human nutrition and health. Further, though pilot experiments with purified BMOs in adults have not yet demonstrated generalizable changes to GI microbial populations (117), future work in infants may be more promising as recent *in vitro* experiments with BMOs are more promising (118, 119).

Complex and hybrid *N*-glycans found in bovine mature milk and colostrum may also provide a source of prebiotic glycans that can be selectively utilized in a fashion similar to HMOs/BMOs (120). Further, the conjugation of these *N*-glycans to milk proteins enable different strategies for their recovery. Protein separation from lactose and subsequent treatment to separate *N*-glycans from their protein conjugates may offer a potentially attractive avenue to purification of these glycans (121). Thus, *N*-glycans derived from bovine colostrum, which is exceptionally rich in *N*-glycosylated proteins (122), may be a potent source of bioactive glycans to serve as prebiotic substrates. Extensive characterization of complex *N*-glycans derived from bovine milk proteins abundant in colostrum now shows that these *N*-glycans are highly selective for certain bacteria in the adult GI microbiome. The bacteria able to access these glycans are further restricted, relative to larger repeated polymers of less complex oligosaccharides which are limited to select *Bifidobacterium* species (e.g., *Bifidobacterium longum* subsp. *infantis*). Some strains of these species have been associated with diminished enteric inflammation and improved GI barrier function in humans (123, 124). Bovine colostrum is also a potential source of anti-infective glycans and recent work provides evidence for the anti-infective activity of oligosaccharides sourced from bovine colostrum against a highly invasive strain of *C. jejuni* (125).

CLINICAL APPLICATIONS OF BOVINE COLOSTRUM

Body Composition and Exercise Performance

The first study investigating the role of colostrum supplementation in exercise performance was completed in 1997 and showed marked improvements on explosive muscle power and increased concentration of immunoglobulins in serum (35). This finding is relevant given intense physical

activity can suppress immunity several hours after training (126). Subsequent, well-controlled studies in comparison to whey protein concentrate have demonstrated significant improvements in lean body mass and weightlifting performance (127), in athletic performance among male and female athletes (128), speed in elite cyclists with dose-dependent effects (129), and in runners for recovery (130). Duff et al. (131) indicated that bovine colostrum supplementation (60 g/d of colostrum) on male and female older adults during resistance training is beneficial for increasing leg press strength and reducing bone resorption in comparison to whey protein complex supplementation. Improvement in the upper body strength, muscle thickness, lean tissue mass, and cognitive function were noted for colostrum supplemented group as well as whey protein treated group (131).

Despite this progress, the exact mechanism behind these marked improvements is not fully elucidated. As human studies typically use whey protein with similar protein content, observed differences are unlikely to simply be a response to protein digestibility or amino acid supplementation. Given that bovine colostrum immune components are likely not providing passive immunity to the human, it is possible that bioactive compounds and/or their metabolites have a direct effect on the immune system (35, 126, 132–135). There is currently weak support for the potential for bovine colostrum supplementation to improve leukocyte function relating to adaptive immunity (126). While a 33% increase in saliva IgA was noted after supplementation of colostrum at 20 g/day for 2 weeks (134) and a 79% increase in IgA in runners fed 12 g/day for 12 weeks was reported (132), these results were not repeated in other studies (35, 135–140). Further, colostrum supplementation diminished exercise-induced intestinal permeability which was replicated in *in vitro* culture models of intestinal epithelial cells (141). Considering the safety profile and generally positive past research from well-controlled studies, further research is warranted to understand the underlying mechanism and explain inter-individual variations and unexplored discrepancies between the growing number of studies on colostrum supplementation in regards to body composition and athletic performance.

NSAIDs Induced GI Inflammation and Permeability

Non-steroidal anti-inflammatory drugs (NSAIDs) are the most common prescribed medicine and used for the symptomatic treatment of acute pain, chronic inflammatory, and degenerative joint diseases (142, 143). NSAIDs can cause gastric and intestinal damage such as peptic ulceration and injury to both the small and large intestine. Complications from NSAIDs use include increased intestinal permeability with protein and blood loss, and also stricture formation (142, 144). Approximately 2% of subjects taking NSAIDs experience adverse effects on the GI tract including bleeding, perforation, and inflammation. Acid suppressants and prostaglandin analogs are used to reduce gastric injury induced by NSAIDs, but these are not adequately effective in preventing small intestine injury. Hence, additional avenues for the mitigation of these negative side effects are needed. Some research suggests that colostrum may be an alternative, owing to the composition of growth factors like α-IGF-1, β-IGF-1, transforming growth factor (TGF), and epidermal growth factors (EGF). These growth factors are capable of stimulating the repair process of the GI tract (145) and are complementary to evidence supporting diminished GI permeability associated with exercise (141).

In a clinical examination of bovine colostrum for protection against NSAID-induced enteropathy, seven male volunteers (26–38 years old) who were taking NSAIDs or suffering from conditions likely to affect intestinal permeability (e.g., coeliac disease or previous intestinal surgery), were evaluated for the potential of bovine colostrum to alter intestinal permeability with concomitant indomethacin supplementation. In this crossover study, following an initial baseline permeability assessment, these volunteers were supplemented orally with 125 mL of bovine colostrum or a whey placebo three times daily for seven days. At the end of the trial period, intestinal permeability was reassessed and a 2-week "washout" period was performed between the crossover. Approximately a 3-fold increase in permeability was observed in the participants taking the whey placebo with indomethacin, while no significant increase in permeability was seen in the participants taking co-administration of bovine colostrum with indomethacin (146). In support of these findings, molecular characterization of the mechanism underlying these effects have been documented. Mir et al., (147) demonstrated that bovine lactoferrin can act as a carrier for NSAIDs by binding to these molecules, but with far lower affinity than the protein targets for NSAIDs which suggests that the efficacy of NSAIDs may not be affected by co-administration of a lactoferrin-containing protein source, like bovine colostrum (147). While further studies will be required to demonstrate that these compounds, when co-administered with bovine colostrum, maintain their desired efficacy, there is growing and consistent evidence supporting the potential for the use of bovine colostrum to manage the potential side effects of NSAIDs.

Uses of Bovine Colostrum in Specific Clinical Populations

Bovine colostrum has led to human supplementation trials due to potential for improvement of GI health and integrity. Several conditions related to GI conditions associated with chronic or acute infections have been investigated for the potential of bovine colostrum to ameliorate symptoms associated with these conditions or infections. While the mechanisms behind these findings are difficult to disentangle given the disparate populations and disease etiologies, there are consistent themes related to the improvement of GI symptoms and reduced inflammation associated with each, though not all conditions demonstrate promising avenues for therapy.

Ulcerative Colitis

Ulcerative colitis, an inflammatory bowel disease associated with durable inflammation and ulcers in the colon (148), was investigated as a potential target for a bovine colostrum enema treatment in a small proof-of-concept trial. The authors rationalized this approach given the high concentrations of

antimicrobial peptides, immunoglobulins and growth factors found in colostrum (149). In this pilot study, fourteen patients with active mild to moderate colitis were compared. Eight patients received 100 mL (10% solution) of bovine colostrum and six patients received an albumin placebo twice per day for 4 weeks. Improvement of the symptom score including patient well-being, abdominal pain, rectal bleeding, temperature, anorexia/nausea, bowel frequency, stool consistency, abdominal tenderness and the presence of extra-intestinal manifestations was reported in seven of the eight patients in the bovine colostrum treated groups (149). While this study is small, the findings show a significant reduction in symptom scores and follow up studies in a similar population with a larger sample size may be warranted.

Necrotizing Enterocolitis

Necrotizing enterocolisis (NEC) is one of the most common morbidities associated with preterm birth, and among the chief causes of mortality among infants born preterm (150). Several studies have examined the impact of either human or bovine-derived colostrum on NEC outcomes and development of preterm infants. In one clinical trial of 86 low birthweight infants supplemented with bovine colostrum in a dose of 2 g, four times per day for infants between 1,000 and 1,500 g and 1.2 g, four times per day for those under 1,000 g at birth. No significant differences were observed in the occurrence of NEC, sepsis, or mortality after the administration of bovine colostrum as compared with placebo (151). In a meta-analysis examining the use of bovine and human colostrum among preterm infants, Sadeghirad et al. concluded that the cumulative findings in the literature suggest that neither human nor bovine colostrum had an effect on the incidence of severe NEC, mortality, culture-proven sepsis, feed intolerance, or length (152). The lack of effects is observed on NEC patients due to some limitations such as using commercial bovine colostrum supplement and number of patients is modest (146, 147). Given these findings, it will be difficult to rationalize continued use of bovine or human colostrum with preterm infants for improvements in these outcomes. However, the use of human colostrum in preterm infants should not be curtailed based on these outcomes as other benefits have been demonstrated for preterm infants (153).

Traveler's Diarrhea

Acute infection with enterotoxigenic *Escherichia coli* (ETEC) represents the most common causes of so-called Traveler's Diarrhea, associated with travel to tropic and semitropical regions throughout the world (154). As bovine colostrum plays a key role in protecting the neonatal calf from environmental pathogens via passive immunity and ETEC represents one of the primary causative agents of neonatal calf diarrhea (155), researchers have been interested in determining whether the same effects can be demonstrated in humans at risk of Traveler's Diarrhea.

Using hyperimmune bovine colostrum which is rich in immunoglobulins targeting 14 strains of ETEC, the efficacy and dose response of consuming bovine colostrum in a tablet form (400 mg of bovine colostrum protein) demonstrated a dose-dependent and significant improvement in protecting against the development of diarrhea among volunteers in a double blinded, placebo-controlled ETEC challenge study. A 400 mg serving of hyperimmune bovine colostrum protein administered with a bicarbonate buffer three times daily conferred 90.9% protection when compared to the placebo (156). Bicarbonate buffer contributes to the enhancement the protective effects of hyperimmune colostrum protein in the ETEC challenge experiments, but the difference was not statistically significant. As little as 200 mg consumed three times per day without buffer gave an estimated 58.3% protection from diarrhea symptoms, compared to the placebo group (156).

In addition to ETEC, viruses contribute to a significant proportion of both neonatal calf diarrhea and Traveler's Diarrhea (154, 155). In a double blinded, placebo-controlled study, Mitra et al., (157) reported that consuming three daily servings of 100 mL of hyperimmune bovine colostrum targeting human rotavirus for 3 days conferred a modest but significant reduction in both the duration of diarrhea and the total stool output among male infants 6–24 months of age (157). Similarly, another study reported that purified immunoglobulins from hyperimmune bovine colostrum conferred a similar effect in acute rotavirus infection, supporting these findings (158).

While neither study examined the impact of colostrum from cows which had not been immunized against the target pathogen, a clinical trial examining the differences between hyperimmune bovine colostrum and bovine colostrum among children infected with shigellosis caused by *Shigella dysenteriae* (*S. dysenteriae*) failed to find any improvements among patients relative to the concurrent antibiotic therapy. However, preclinical studies in other biomedical models (e.g., gnotobiotic pigs) have shown promise for hyperimmune bovine colostrum in preventing diarrhea caused by *Clostridioides difficile* (*C. difficile*). Together, these findings may suggest that infectious mechanisms of pathogenesis shape the ability of hyperimmune bovine colostrum to influence disease progression as *S. dysenteriae* invades epithelial cells (159), potentially evading hyperimmune bovine colostrum immunoglobulins, while ETEC and *C. difficile* utilize secreted toxins to induce epithelial damage (160, 161).

FUTURE DIRECTIONS

Given the biological role of colostrum for neonates (8, 162), its documented bioactive components as outlined here, and the potential for development as a functional food or food ingredient. There is a significant interest in developing colostrum as an ingredient to improve the bioactivity of foods and/or their potential health benefits. With a higher protein content and lower lactose concentration, this favorable protein/carbohydrate ratio is also nutritionally attractive and the potential for the future development of the ingredients and constituent fractions of colostrum is promising. However, overcoming processing challenges to separate bioactive fractions from colostrum remains a challenge to both study the mechanisms by which this fluid can act on humans and for practical product development. Future

clinical trials should address current gaps in understanding which populations, such as those with GI disorders, may benefit most from colostrum consumption and whether whole or fractionated colostrum offers the most attractive balance of nutrition and bioactive properties.

AUTHOR CONTRIBUTIONS

SK organized the general content of the paper. AA was responsible for general editing and organizing the authors and also responsible for the two sections of the paper. MK contributed one section of the paper. HD was responsible for writing one section of the paper. AB was responsible for the one section of the paper. ME contributed to a section of the paper. BMH was responsible for the organizing a section. SAF contributed editing and organizing the paper. All authors contributed to the article and approved the submitted version.

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REVIEW
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Well-being Messaging for Mammalian Milks: A Scoping Review

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Having a holistic understanding of research on well-being messaging for milk is vital to allow the optimal communication of the association between milk consumption and various nutritional, physical, and psychological benefits to the consumer. This work is a unique interdisciplinary, scoping review of existing research on well-being messaging for milk. Well-being messages are ways to communicate the broad well-being benefits of specific products to the consumer through information on food content or statements that link a product with favourable components, functions, or well-being outcomes. Leveraging this broad definition, and by proposing a guiding theoretical model that considers well-being messaging as a form of communication, milk well-being messaging literature has been mapped across time, geographical locations, disciplines, and product types. Two hundred forty-six were records included in this review, of which 177 were empirical studies. Studies were disseminated between 1954 and 2019, with 54.9% published after 2011. Food, Agriculture, and Biological Sciences ($N = 109$), Nutrition and Dietetics ($N = 78$), and Medicine, Public Health, and Health Professions ($N = 69$) disciplines have attracted the most publications, with numbers generally increasing in most recent years. The majority of included non-empirical records (69.6%) provide lists of commercially available products carrying well-being messaging and/or regulations on the use of particular well-being messages for milk according to various legislative authorities. Most of the empirical studies were conducted in North America ($N = 71$), West Europe ($N = 52$), and Oceania ($N = 22$), and on plain (i.e., unflavoured) milk ($N = 152$). Whereas, most studied elements of well-being messaging for milk across time, i.e., *message* ($N = 169$), *product* ($N = 141$), *receiver* ($N = 101$), and *context* ($N = 72$) have seen an increasing number of studies in recent years; *sender* ($N = 51$) and *medium* ($N = 27$) have been even less studied in the past four years. A more detailed analysis of research trends in each element of well-being messaging is reported. The research highlights immediate and strategic knowledge gaps that need further attention from researchers and/or policymakers in order to improve the "messaging" of well-being benefits of milk consumption to the consumer.

Keywords: well-being, food label, health claim, health communication, milk, scoping review, well-being messaging

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INTRODUCTION

Whether looking at ancient mythologies or the documented history of mankind, there are few food products that appear consistently, milk is undoubtedly one of them. Milk, exclusively defined as the normal mammary secretion obtained from milking of mammals, including but not limited to bovine (1), for millennia, has been regarded as an integral part of human diet in most parts

of the world. It has most commonly been regarded as a nutritious food with various health benefits. Despite some polarising debates emerging in recent years, mounting evidence from various research disciplines continues to support the unique nutritional value of milk (1–3) and an association between milk consumption and many physical and also psychological benefits. A non-exhaustive list of the well-being domains that milk consumption has been positively associated with reduced incidence of colorectal, bladder, and gastric cancer reduced risk of obesity, diabetes, cardiovascular disease, frailty, and sarcopenia, and improved metabolism, bone health, gut function, and cognitive performance (1, 4–6).

The association between milk consumption and well-being, no matter how scientifically established, can only reach the consumer if it is communicated accurately and effectively to the public. Informing consumers of the health and nutritional value of milk has, indeed, been shown to influence milk consumption behaviour, purchasing behaviour, and willingness to pay (7–11). Food labels have, for a long time, been considered to inform the consumer about well-being effects of food products (12, 13). Surveys in Western countries suggest that food labels are relatively popular sources of information among the general population and more so among certain market segments, including women, higher-income earners, younger consumers, Caucasians, weight-conscious, or worried-well (see 12, for a review). Delivered at the point of choice, purchase, or consumption, food labels can be of various types (e.g., mandatory vs. voluntary), frames (e.g., promoting vs. warning), designs (e.g., pictorial vs. textual), content (e.g., nutrition, health, or risk reduction), and strengths (e.g., qualified health claims vs. marketing information). Fat content of milk, to give an example, has traditionally appeared on a package (e.g., "low fat," "skim milk," and "full cream"). Recent advancements in dairy science and technology (e.g., developing functional foods with specific health effects), as well as marketing and retailing approaches (e.g., growing popularity of online supermarkets or social media marketplaces), have certainly added more sophisticated front-of-pack (FOP) profiling systems and QR codes to traditional food labels. These new forms of information, while ultimately performing as means to communicate well-being messages of a product to the consumer, have commonly been targeted as separate lines of research across various disciplines and, sometimes, with terminology and scope that do not include other forms of messaging. Hence, despite existing reviews of literature on either nutrition labels (14), health-related claims (15), point-of-choice messaging (7), and FOP labels (16) for food products, no effort has, to date, been made to map relevant literature on milk well-being messaging regardless of the type, frame, design, content, strength, or medium of a message.

This review aims to bring together a seemingly diverging literature around what is essentially *well-being messaging* for milk regardless of species. This is achieved through the provision of an all-encompassing definition of well-being messaging and proposition of a guiding theoretical model that considers well-being messaging as a form of communication. Together, these make it possible to map the literature on well-being messaging for milk across time, geographical locations, disciplines, and product types. The research, therefore, highlights immediate and strategic knowledge gaps that need further attention from researchers and/or policymakers in order to improve "messaging" the well-being benefits of milk consumption to the consumer.

Well-being Messaging

While a commonly used term in the literature is "health," well-being brings about a broader connotation that covers not only the state of being physically healthy but also potential psychological and social benefits that are offered by consuming food. Fittingly, well-being, as a multifaceted concept, is defined as a way of living that directs one toward the highest levels of feeling good and functioning well in various biopsychosocial domains (17–19). Interestingly, prior research highlights the association between milk consumption and various psychological and social domains (20–23), hence justifying the need for the use of the term "well-being" instead of "health" in relation to milk.

With a definition of well-being stated, well-being messaging can largely be considered as communicating well-being-related information. To provide a working definition, however, well-being messaging is defined as ways to communicate to the consumer the broad well-being benefits of specific products through information on food content or statements linking the product with favourable components, functions or well-being outcomes [for a comparison, see (24)]. Being broad enough, this definition fits the purpose of this research to include instances of well-being messaging for milk regardless of type, frame, design, content, strength, or medium.

Theoretical Framework

Review studies benefit from a predetermined conceptual framework, which is anticipated to best encompass the broad questions of interest (25, 26). Having a conceptual framework is particularly useful as a basis for scoping the boundaries of the review (e.g., inclusions and exclusions), initial coding, and categorising the existing evidence. A framework also helps to identify patterns, trends, and themes in prior research and thereby highlights knowledge gaps in the literature (25–27). This so-called "best fit" framework has also been suggested to offer promising responses to practise-relevant and policy questions about the topic of interest (27).

For the purpose of this review, Lasswell's communication model, a classic model of communication, has been chosen as a framework for mapping out milk well-being messaging literature. According to this model (28), any act of communication is comprised of five essential elements: sender (i.e., who said it), message (i.e., what was said), medium (i.e., through what channel it was said), receiver (i.e., to whom it was said), and outcome (i.e., what was the desired outcome). Fittingly, a well-being message is a *message* that is sent by a *sender* (e.g., food manufacturer) through a specific *medium* (e.g., on or off the food package) to facilitate an anticipated *outcome* (e.g., increased well-being awareness or willingness to purchase) in *receiver(s)* (e.g., a particular market segment).

To the extent that Lasswell's original model is criticised for ignoring the context in which the communication takes place, a limitation that has been addressed in subsequent

theories of communication [e.g., (29)], a well-being message, as the specific communication type of interest here, is also highly contextualised. As such, legislative zones, geographical markets, and/or selling venues are likely to influence (or even enforce) the decision of a manufacturer with regard to various aspects of the well-being messaging. Also, despite the fact that "medium," in accordance to Lasswell's model, can broadly include the product itself as a channel that delivers the message, to provide further practicality and clarity around categorisation of published research and identification of knowledge gaps, product, and medium have been considered as separate elements of communication in this review.

Adding context and product to the original model, it is proposed that well-being messaging studies conducted in any discipline are ultimately engaged in investigating one or more of these major elements: product, message, sender, receiver, medium, context, and outcome. The remainder of this section provides a working definition of each element together with instances of each that have been incorporated in categorising records in this scoping review.

Product is milk (i.e., including milks of all mammalian origins, but excluding plant-based imitation milks) and other milk drinks included within the scope of this review. Product-related research thus includes any instances of product characteristics (e.g., price, total fat, sugar, calories, and other nutrient contents, size, packaging design, or material) when studied in association with well-being messaging.

Message simply refers to the information that is conveyed through the well-being message. Hence, studies that have investigated the content and/or the graphical and verbal design of well-being messaging have been categorised under this element.

Sender denotes the source of the well-being message. The sender is usually the food manufacturer. As such, any piece of evidence examining manufacturer-related factors (e.g., brand, image, size, and country of origin), as well as organisation-related decisions and procedures involved in (or affected by) development, design, use, and/or (re-)evaluation of well-being messaging, has been categorised under this element. In addition, endorsements and verifications of the well-being message by sources other than the manufacturer have also been considered as instances of sender related.

Receiver of the well-being message is generally the consumer. Well-being messages, however, are sometimes used to target a particular segment of the market, determined by factors such as consumer age, particular health concerns, socioeconomic status, or social group. Studies that have investigated consumer-related factors (e.g., demographics, diet and eating habits, lifestyle, health status, and bodily measures), whether individually or in order to create clusters of consumers, in relation to well-being messaging, have thus been categorised as receiver related. It is important to note that records wherein participant information is only mentioned in descriptive statistics and not in association with well-being messaging have not been considered as instances of receiver-related research.

Medium refers to the exact channel of well-being message delivery. While the usual medium of delivering well-being messaging is the food package, well-being messages are sometimes delivered off-the-pack (e.g., TV commercials, online shopping platforms, or supermarket displays). Instances of empirical research analysing placement of well-being message, delivery format and design, point of choice delivery, marketing strategies to boost well-being messaging, and advertisements have all been considered as medium-related research.

Contexts in which well-being messages are used may be analysed at various levels, e.g., selling point, time and date, geographical market, and legislative zone. Factors relevant to each level, when analysed in relation to well-being messaging, have been counted as instances of context-related research. It should, however, be noted that investigations of self-identified social groups (e.g., religious or ethnic group) are considered instances of receiver-related research.

Well-being messaging aims at, and possibly delivers, various *outcomes*. Food manufacturers, for instance, use well-being messages with the ultimate goal of increasing their market sales, perhaps through enticing segments in the market who would most likely benefit from consuming the product. This outcome, when considered from a consumer point of view, is usually translated as purchasing and consumption behaviour. Various outcomes of interest in milk well-being messaging research, whether related to design, delivery, or perception of well-being message or as a result of utilising well-being messaging, have been included here.

It is also worth noting the inclusive approach that has been taken in categorising evidence under the elements listed above.

Research Questions

Within the presented theoretical framework and stated definition of well-being, this review aims to answer the following research questions (RQ):

RQ1: What are the identifiable research trends (across scientific disciplines, time, geographical locations, product type, and research methods) regarding well-being messaging for milk?

RQ2: Akin to the proposed conceptual framework, what well-being messaging elements have received more attention across scientific disciplines, time, and geographical locations than others?

RQ3: What have been the specific areas of interest within each well-being messaging element across time and for various product types?

RQ4: What have been the specific outcomes of interest within each scientific discipline?

METHODS

Nature, scope, and existing knowledge around a scientific enquiry are the keys when deciding the correct approach to address it. Given the form of our research questions, multidisciplinary nature of the area, the expected heterogeneity of research methods, and inability to find comprehensive reviews of the topic through a preliminary search, a scoping review was chosen as the appropriate method. A scoping review is "a type of knowledge synthesis, following a systematic approach to map evidence on a topic and identify main concepts, theories, sources, and knowledge gaps" (30, p. 467). Scoping reviews are

mainly aimed at painting a big picture of the extent, range, and pattern of research activity in a topic area of a particular complex, interdisciplinary, or heterogenous nature, clarify key concepts and identify knowledge gaps in the existing literature, and determine the value and potential scope of undertaking a systematic review (30, 31). The methodology and the reporting agenda in this study are adapted from suggestions by Pham et al. (31), Arksey and O'Malley (32), Levac et al. (33), and Tricco et al. (34) in an effort to follow the best practise guidelines in the literature while accommodating the specificities of our research.

Identification of Relevant Studies

Relevant literature for this review was extracted from a database of research studies on well-being messaging for food products systematically developed by the authors. Relevant studies cited, but not originally included in the database, were also added to the pool of identified literature for final screening. An account of how this comprehensive database was developed and relevant studies identified is provided below.

Database of Well-being Messaging Studies for Food Products

The database systematically mapped interdisciplinary literature around well-being messaging for food products, including but not limited to milk. It was developed following guidelines in the literature (35, 36) and according to a detailed preregistered protocol, which is accessible *via* the open-science framework repository (37).

Data Sources and Search Strategy

EBSCo (Academic Search Complete, Art and Architecture Complete, Business Source Complete, Cinahl, EconLit, Education Source Complete, MEDLINE, PsycInfo, and PsycArticles), Scopus, Web of Science, Cochrane, and ProQuest were searched between 23–31 December 2019 for academic journals, early access, reviews, conference materials, editorial material, books, e-books, book chapters, and dissertations published in English up to and including December 2019. Given the interdisciplinary nature of the enquiry, the data sources were selected to be comprehensive and to cover a broad range of disciplines. The search query of titles, abstracts, and keywords, while being tailored to the specific requirements of each source, consisted of the following sets of terms: all combinations of type terms (e.g., "health," "wellness," and "well-being") and communication terms (e.g., "claim," "label," and "message"), topic terms (e.g., "food," "product," and "consume"), but excluding irrelevant terms (e.g., "insurance," "health information technology," and "health monitor"). The search query was informed by *a priori* knowledge, examining recent relevant systematic reviews, and following pilot searches. The search query for Scopus is provided as an example in the preregistered protocol (37).

Eligibility Criteria

Studies were included if they were: (i) document types limited to academic journal articles, doctoral dissertations, book and book sections, indexed reports, and conference papers; (ii) involving any research designs and methodologies, including empirical and non-empirical records (e.g., reviews, theoretical pieces, letters, and opinion papers); (iii) records with full texts obtainable through institutional holdings available to the authors or published in open-access outlets; (iv) records with full text in English (It should be noted that, despite excluding the records with non-English full texts, no limitations have been set regarding the country in which the study was carried out or where it has been published); (v) records attending to well-being messages of all types and formats, with the notable exceptions of an ingredients list and a nutrition information table; (vi) involving well-being messaging for pre-packaged food products, excluding alcoholic drinks, tobacco products, unpackaged food, menus, drugs, dietary, nutritional, herbal, and medical supplements, nutraceuticals, and the like.

Citation Management

Citations were imported into Mendeley Desktop 1.19.4, and duplicate citations were removed manually. Citations were then imported into Rayyan data management software for screening (38). Finally, selected citations were imported into Endnote X9 with full texts added automatically *via* the "find full text" feature, or manually. Further duplicates were removed when found during the screening process, manually, or using Endnote X9.

Screening Process

Records were screened for eligibility in Rayyan, first based on title only, and then abstract and keywords. In rare cases, full texts were consulted during the prescreening step. Following prescreening, a further screening examined document-type eligibility, full-text language, and full-text accessibility on Endnote X9.

The screening process, conducted by the first author, followed a predetermined procedure (outlined in detail in the preregistered protocol) to mitigate assessor bias. Specifically, after transferring search results to Rayyan, a random subsample of 100 results was created by the first author and transferred to a separate pilot screening project in Rayyan. This subsample was blindly screened based on title, abstract, and keywords by both authors. The first screening round resulted in 77% inter-rater agreement and did not reach the predetermined 80% threshold. Hence, after discussing cases of disagreement, a second screening was conducted and resulted in 93% agreement.

The procedure outlined above resulted in 3,337 records being included in the general well-being messaging database (see **Supplementary Figure 1**).

Identifying Relevant Studies for the Scoping Review

To identify studies focusing on well-being messaging for milk, the database was searched for the following three steps: (i) full texts of all included records were prescreened to separate searchable and unsearchable PDF files; (ii) searchable PDF files (N = 3,255) were automatically searched using the search function of Adobe Acrobat Reader for variations of the term "milk" (e.g., "milks," "milkshake," and "milkfat"). Records including any variation of "milk" were considered for further screening; (iii) all unsearchable PDF files (N = 82) were also considered for further screening.

Additional studies relating to well-being messaging for milk, whether cited in-text or in reference lists of the database records, were sourced and added to the pool of records for further screening ($N = 83$).

In total, 3,420 records were identified for screening for the scoping review.

Scoping Review Study Selection Process

Additional Eligibility Criteria

As well as the well-being messaging database eligibility criteria (listed in section Eligibility Criteria), the following additional criteria were added: (i) records pertaining to well-being messages for all types of pre-packaged ruminant milks, including pourable, powdered, and flavoured milk, functional non-fermented milk, and milkshake, but excluding infants and toddlers milk formula, nondairy milk substitutes, and fermented milk drinks were included; (ii) records in which findings exclusive to milk or other product types of interest were not separated from other products (e.g., dairy products, milk products, milk and other dairy products, milk and other soft drinks, and milk and other milk alternatives) were excluded in the screening process.

All identified records were imported into Endnote X9 and full texts were screened against the eligibility criteria. To reduce assessor bias, a random subsample of records in the well-being messaging database (98 records out of 3,255 searchable PDF files) was blindly searched for and screened for eligibility by both authors. Inter-rater agreement of 91% was achieved (> the predetermined 80% threshold), cases of disagreement were discussed, and the first author continued with screening. The screening consisted of two steps. The first step (i.e., prescreening) excluded 3,073 searchable PDF records based on the fact that an automated text search for any variation of "milk" did not return eligible results. This step identified any mentions of milk and its variations anywhere in the record and made it possible to evaluate the eligibility of the record without the need to go through the full text. Cases for which a clear decision could not be made *via* this process were considered for further screening (i.e., the second step).

The second step involved screening the remaining 347 records that either the file format did not allow for automated text search or a final decision for eligibility was deemed difficult after the prescreening step. Either case, the full text was manually screened for relevance and eligibility against the inclusion and exclusion criteria. This resulted in 246 records (the list obtainable on request *via* feast@massey.ac.nz) for inclusion in this scoping review (see **Figure 1**).

Charting the Data

Data charting was completed with an *a priori* coding framework (see **Supplementary Table 1**) focused on time of publication, publication outlet, and its associated scientific discipline(s), record type for both empirical and non-empirical records, and research method, study design, product type, country(-ies) of study, well-being messaging elements of focus (i.e., product, message, sender, receiver, medium, and context), outcome(s) of interest only for empirical records. Non-empirical records, however, were also accompanied by a brief summary of content and were classified as one of the following publication types: reviews (which include meta-reviews), case reports, conceptual pieces, or opinion papers. Given the multidisciplinary nature of the review and variety of designs employed, "study design" was dropped from the framework as accurate identification and reporting were deemed to be difficult. Publication years and geographical locations were categorised into time periods of 1954–2000, 2001–2005, 2006–2010, 2011–2015, and 2016–2019; and geographical regions as North America, Latin America, West Europe, East Europe, West Asia, East Asia, Africa, and Oceania following Maddison (39). Scientific discipline(s) of each record were determined *via* journal-associated disciplines identified *via* Scimago Journal and Country Rank (https://www.scimagojr.com) and further grouped into 19 disciplines (see **Figure 3**). Records published in outlets other than academic journals were classified as "unspecified" discipline. Research methods were classified as quantitative, qualitative, and mixed methods. Product types were classified as plain (i.e., unflavoured) milk (fluid), plain milk (powder), flavoured milk, milkshake, others. Unspecified product types were classified as plain milk (unspecified). Finally, it is worth noting that, for records including empirical and non-empirical sections, multiple studies, various methods, or more-than-one-product type or a well-being messaging element of focus, parts that met the eligibility criteria were used for charting the data. Similarly, among the variables of study, only those studied in connexion with well-being messaging were used to chart the data. Hence, study participant information, for instance, was only considered to be an example of a receiver-related study when investigated in association with well-being messaging.

RESULTS

General Trends

Out of the 246 records included in this review, 177 records were classified as empirical (72%), and 69 as non-empirical (28%). The ratio of empirical to non-empirical records, however, has been consistently increasing in recent years (**Figure 2**). While only 63.2% of records published between 1954 and 2000 were empirical, the percentage increased to 80.9% between 2016 and 2019.

Empirical records were composed of 167 journal articles (94.3%) with the remainder book chapters (1.1%), conference papers (1.7%), PhD theses (0.6%), and published indexed reports (2.3%). The majority of empirical records took a quantitative approach (87%). Qualitative and mixed methodologies were used in 7.9 and 5.1% of the empirical records, respectively. With regard to product type(s) of interest, plain milk was the most commonly studied product type (152 records) compared with flavoured milk (31 records), milkshake (8 records), and other milk drinks (7 records). Among plain milk records, however, powdered milk has received much less attention (5 records) compared with fluid milk (56 records). It is worth noting that, in a considerable number of the records investigating plain milk, the format (fluid vs. powder) of the product was not specified (91 records). It should also be noted that some records had more than one type of product; hence, the counts presented are mutually inclusive. See

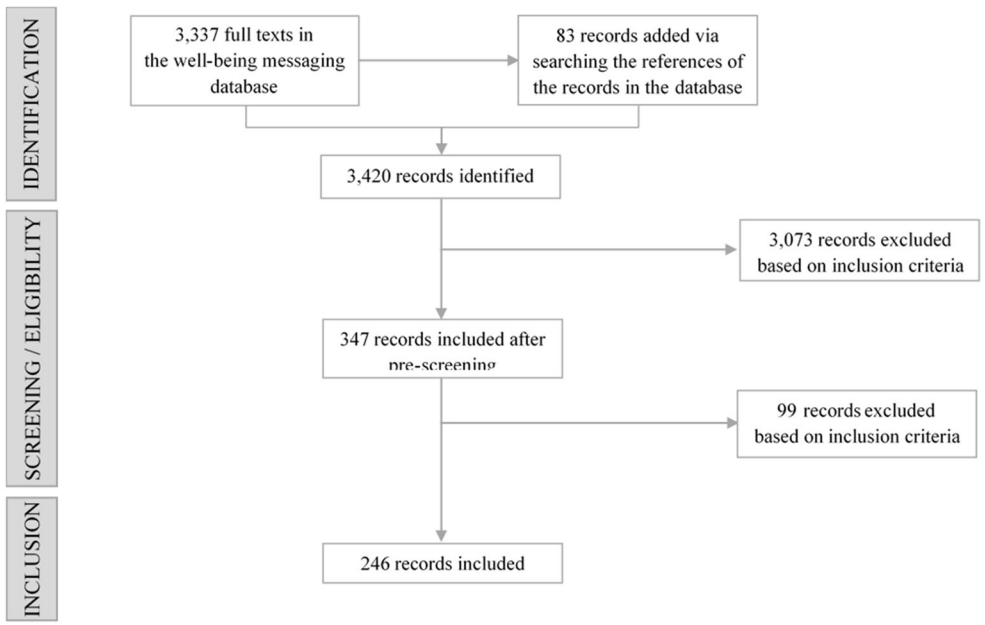

FIGURE 1 | A search flowchart resulting in the final 246 records included in this review.

Table 1 for a more detailed breakdown of the empirical records by record type, research method, and product type.

Non-empirical records comprised 51 journal articles (73.9%), with the rest being book chapters (20.3%), books (2.9%), and conference papers (2.9%). Review studies, including narrative, systematic, and meta-reviews, accounted for almost 87% of the entire non-empirical records included in this study, with case reports, conceptual pieces, and opinion papers, each representing up to 4.3%. **Table 1** provides a detailed breakdown of non-empirical records by record type and publication type.

A further breakdown of records by time and academic discipline(s) of publication is presented in **Figure 3**. While accepting that each record may have been assigned to more than one academic discipline, the following notable trends could be observed: (i) Food, Agriculture, and Biological Sciences, Nutrition and Dietetics, Medicine, Public Health, and Health Professions, respectively, have attracted the most publications, with numbers generally increasing in most recent years; (ii) Economic, Econometrics, and Finance, Marketing, Management, and Accounting, Psychology, and Biochemistry, Genetics, Micro- and Molecular Biology have each been involved in at least 20 publications across time, albeit with somewhat different temporal trajectories; (iii) while publication numbers in several disciplines, e.g., Energy and Environmental Sciences, and Social Sciences (Other) have been growing in the past 15 years, disciplines like Engineering and Chemistry, Communication, and Law have not been involved in publishing research in well-being messaging for milk in the past few years.

Overview of Non-empirical Literature

While this review is set to provide a detailed account of empirical records, this section features a brief yet unique overview of non-empirical records, which serves three key purposes: First, to inform the reader about the nature, scope, subject, and methodology of previously published reviews; second, unlike disciplines, such as Communication, Neuroscience, and Social Sciences, where the included records were all classified as empirical, milk well-being messaging research in Law was confined to non-empirical studies, including two original pieces (case report and opinion paper), which would be of interest for readers within the discipline or across other disciplines; third, the majority of included non-empirical records (69.6%) provided lists of commercially available products carrying well-being messaging and/or regulations on the use of particular well-being messages for milk according to various legislative authorities, which are both of key interest to industry and policy-makers.

Given the above, a summary of non-empirical records has been provided in the (**Supplementary Table 2**) that can be used to assess record and publication types, and the main themes of non-empirical research throughout the time span of this review. It should be noted that none of the reviews provided either a scoping review of well-being messaging for milk or an interdisciplinary approach to the topic, hence adding to the importance of this research as a unique contribution to the existing literature. Also, a closer look at the most recent nonempirical records (i.e., 2016–2019), it is clear that: (i) 10 out of 14 records published in this period were journal articles with the rest being book chapters; (ii) except for one case report published in 2018, all other non-empirical records have been categorised as reviews; (iii) the main theme of the included records content-wise related to food-related regulatory systems in different legislative zones, especially with regard to well-being messaging for milk (8 records), lists of commercial milk across the globe carrying well-being messaging (3 records),

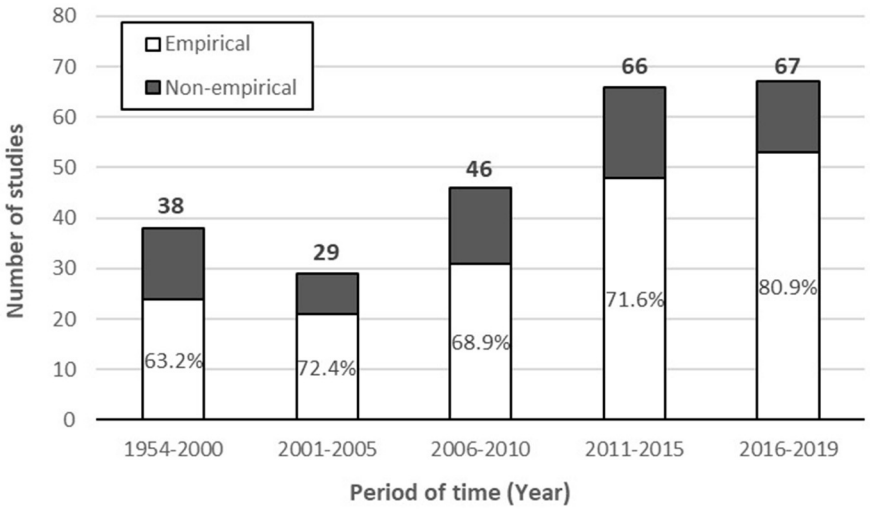

FIGURE 2 | Prevalence of empirical and non-empirical studies of well-being messaging for milk during each period (i.e., 1954–2000, 2001–2005, 2006–2010, 2011–2015, and 2016–2019). Numbers over each bar represent the total count of studies during each period. Empirical studies as a percent of total have been indicated inside the bars.

TABLE 1 | A summary of empirical and non-empirical records by record type, method, product type, and publication type.

Empirical (177)			**Non-empirical (69)**	
Record type	**Method**	**Product type***	**Record type**	**Publication type**
Journal article (167)	Quantitative (145)	Plain milk (fluid) (46)	Journal article (51)	Review (42)
		Plain milk (powder) (4)		Case report (3)
		Plain milk (unspecified) (71)		Conceptual piece (3)
		Flavouredmilk (29)		Opinion paper (3)
		Milkshake (8)	Book Chapter (14)	Review (14)
		Others (6)	Book (2)	Review (2)
	Qualitative (14)	Plain milk (fluid) (1)	Conference paper (2)	Review (2)
		Plain milk (powder) (1)		
		Plain milk (unspecified) (11)		
		Others (1)		
	Mixed methods (8)	Plain milk (fluid) (3)		
		Plain milk (unspecified) (5)		
		Flavouredmilk (1)		
Book chapter (2)	Quantitative (1)	Plain milk (fluid) (1)		
	Mixed methods (1)	Plain milk (unspecified) (1)		
Conference paper (3)	Quantitative (3)	Plain milk (fluid) (3)		
		Flavouredmilk (1)		
Thesis (1)	Quantitative (1)	Plain milk (fluid) (1)		
Indexedreports (4)	Quantitative (4)	Plain milk (fluid) (1)		
		Plain milk (unspecified) (3)		

Counts are shown between brackets.
**Some records investigated more than one type of product.*

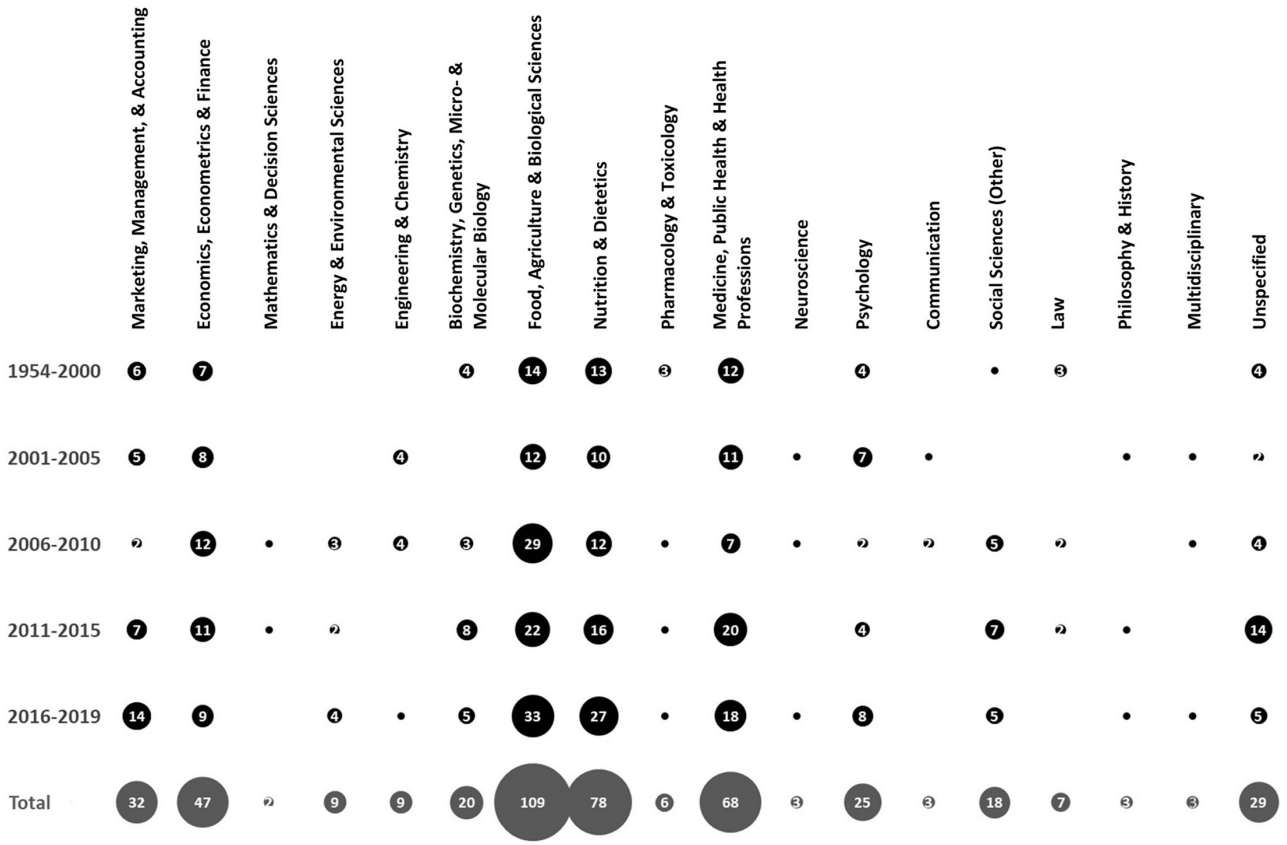

FIGURE 3 | Prevalence of research in each discipline during each period (i.e., 1954–2000, 2001–2005, 2006–2010, 2011–2015, and 2016–2019) and in total (i.e., between 1954 and 2019). Numbers in bubbles specify the prevalence. Note that bubbles with no number represent only one record.

impacts of well-being messaging, among other interventions, on milk consumption and willingness to pay for milk (3 records), and a review of product- and consumer-specific characteristics influencing the impact of well-being messaging for food products, including milk.

Overview of Empirical Literature

Comparing various well-being messaging elements (product, message, sender, receiver, medium, and context), however, indicated that the increase in number of empirical studies has not been consistent across them. Whereas, studies of well-being messaging for milk-investigating product, message, and receiver have all increased over time, studies that focus on sender and medium are now less prevalent, at least in the past 4 years (see **Figure 4**). Additionally, the number of investigations of each element across time, i.e., *product* ($N = 141$), *message* ($N = 169$), *sender* ($N = 51$), *receiver* ($N = 101$), *medium* ($N = 27$), and *context* ($N = 72$), highlighted a potential gap in knowledge about medium, sender, and even context, compared with the other elements. This knowledge gap seemed to be prevalent across all disciplines interested in milk well-being messaging, albeit with some noticeable discrepancies between disciplines (see **Figure 5**). Particularly, while product and message have disproportionately been the centres of focus in Nutrition and Dietetics, receiver and, to some extent, context have also received some amount of interest in disciplines such as Food, Agriculture, and Biological Sciences, Medicine, Public Health, and Health Professions, Economic, Econometrics, and Finance, and Marketing, Management, and Accounting.

Considering geographical regions, North America, West Europe, and Oceania with 71, 52, and 22 total records, respectively, could be highlighted as hotspots for empirical research in well-being messaging for milk across the time span of this review (see **Figure 6**). Nevertheless, the past 4 years showed a considerable drop in study numbers in North America. Also, the most recent 10–15 years have witnessed the first few studies emerging in regions such as Latin America, West Asia, and Africa. Most notably, however, East Asia, including populous countries, such as China, India, Indonesia, Pakistan, Bangladesh, Japan, Philippines, and Vietnam, accommodating about half of the World's population, has only been subject to 13 empirical enquiries across time and with no studies published before 2006. With regard to the particular well-being messaging elements of interest, message, and product have been most studied regardless of the geographical region (see **Figure 7**). Interestingly, context in East Asia and East Europe, two generally less-studied regions, has been included in more than half of the empirical studies of well-being messaging for milk in the region. Also, the receiver of the

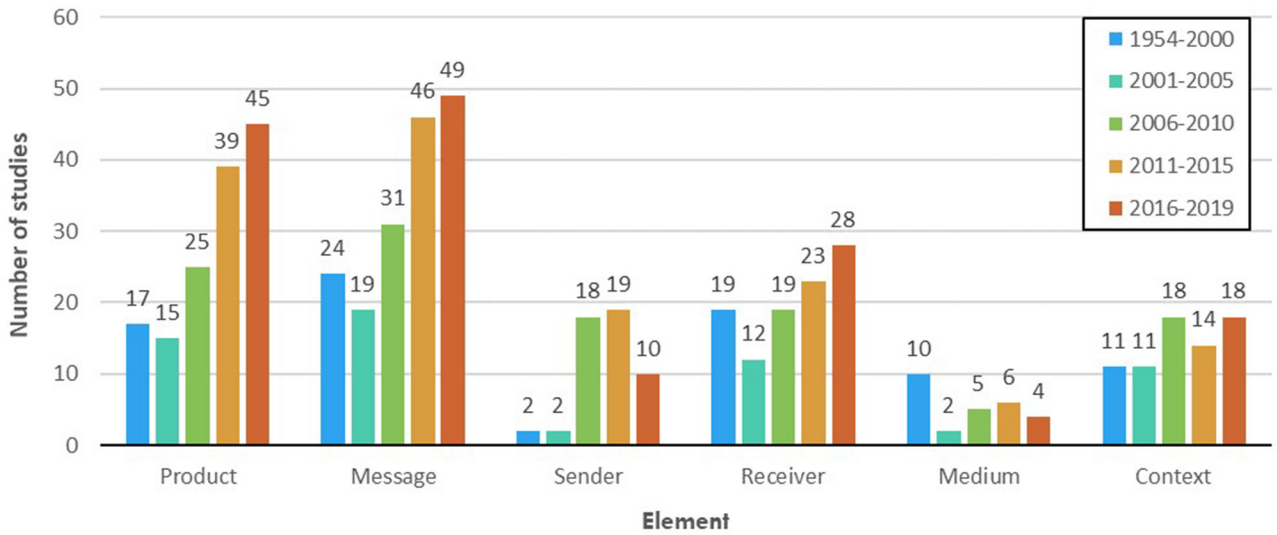

FIGURE 4 | Prevalence of empirical studies of each element (i.e., product, message, sender, receiver, medium, and context) during each period (i.e., 1954–2000, 2001–2005, 2006–2010, 2011–2015, and 2016–2019).

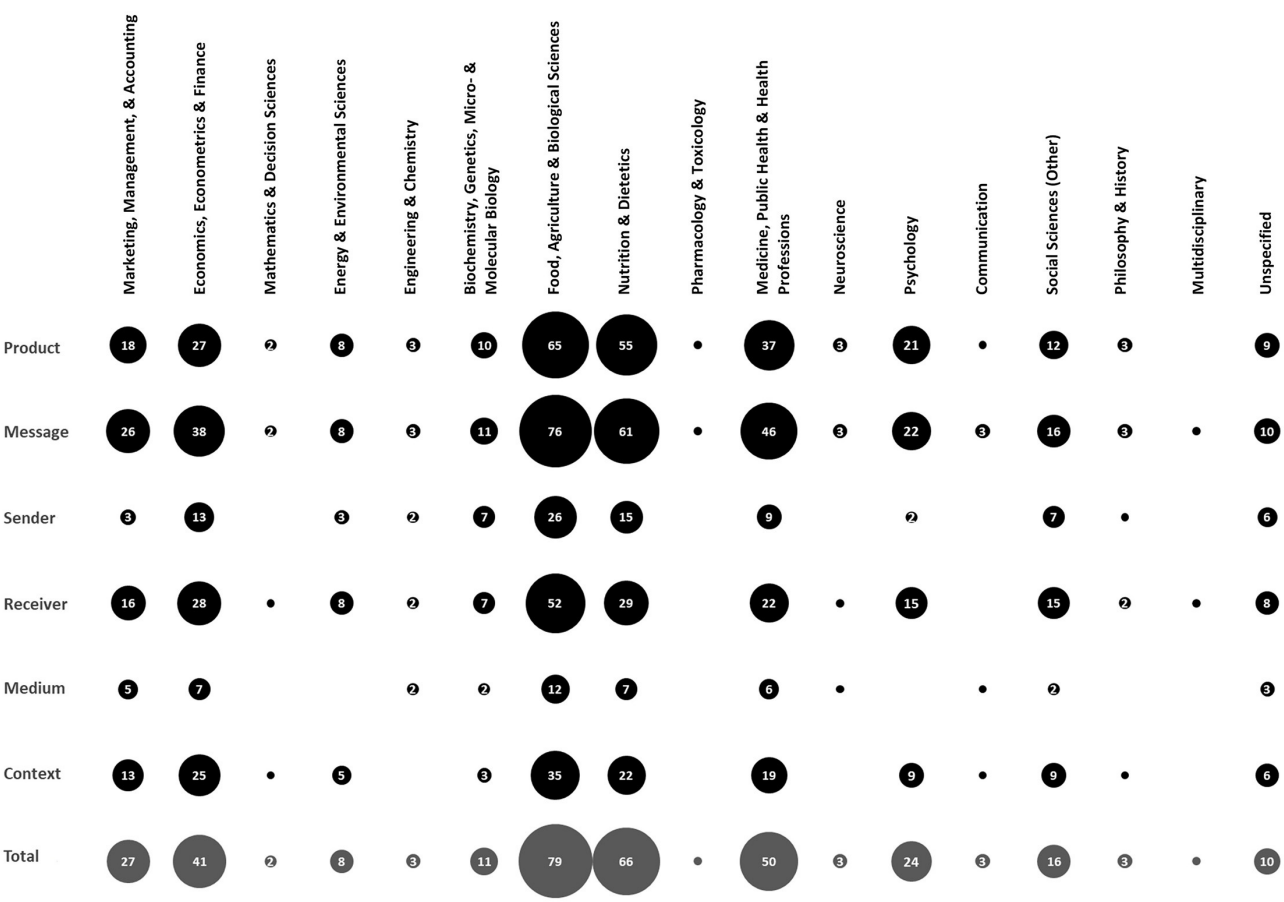

FIGURE 5 | Prevalence of empirical studies in each discipline related to each element (i.e., product, message, sender, receiver, medium, and context) and in total. Numbers in bubbles specify the prevalence. Note that bubbles with no number represent only one record.

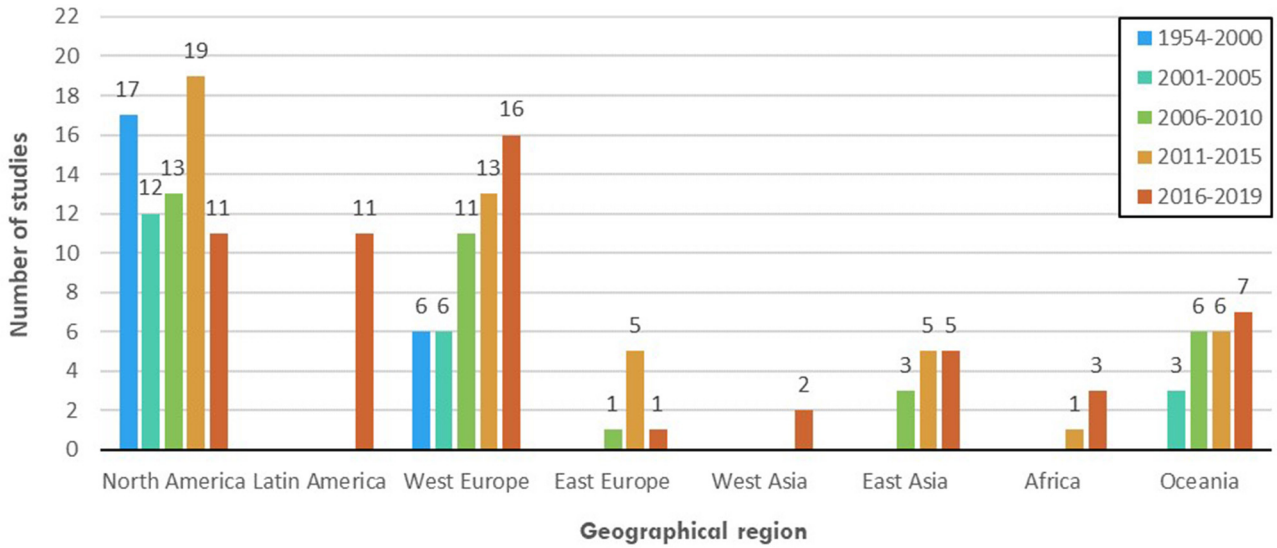

FIGURE 6 | Prevalence of empirical studies in each geographical region (i.e., North America, Latin America, West Europe, East Europe, West Asia, East Asia, and Oceania) during each period (i.e., 1954–2000, 2001–2005, 2006–2010, 2011–2015, and 2016–2019).

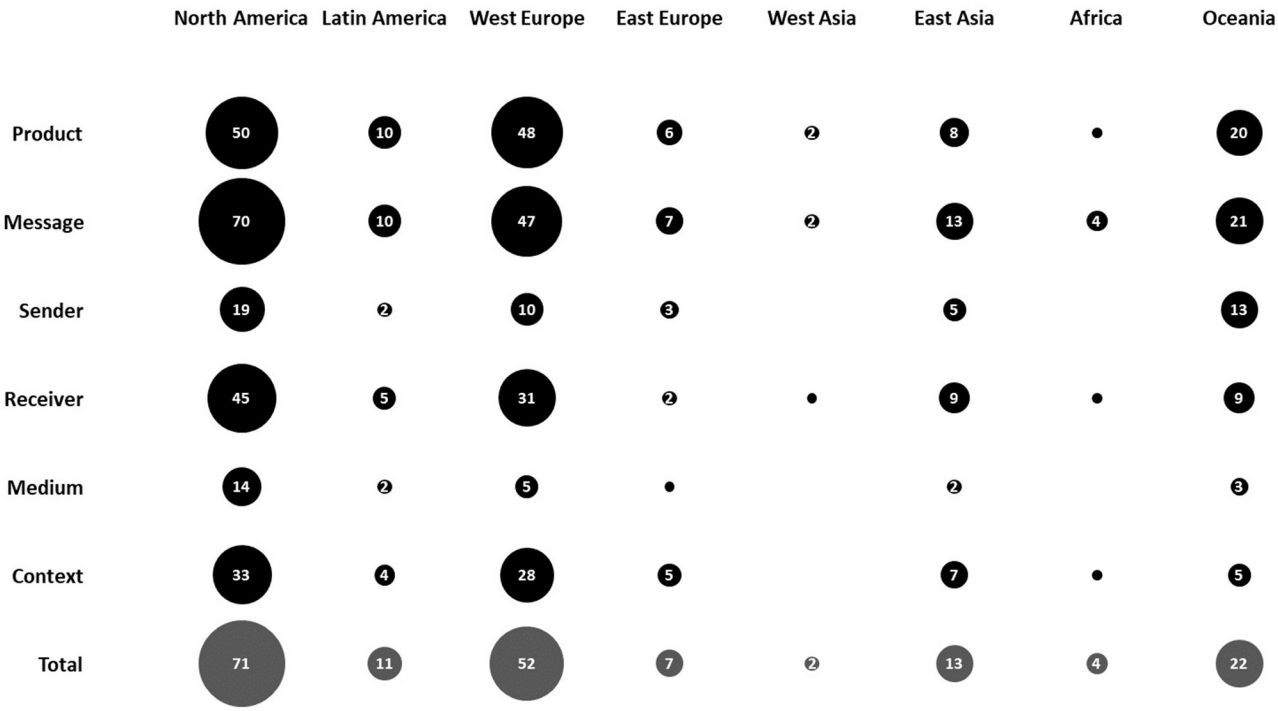

FIGURE 7 | Prevalence of empirical studies in each geographical region (i.e., North America, Latin America, West Europe, East Europe, West Asia, East Asia, and Oceania) related to each element (i.e., product, message, sender, receiver, medium, and context) and in total. Numbers in bubbles specify the prevalence. Note that bubbles with no number represent only one record.

well-being messaging has been an important element of interest, even more so than product, in studies conducted in East Asia. Finally, sender, while being understudied in general, has featured in 13 out of 22 total studies of well-being messaging for milk in Oceania, considerably more often than in other world regions.

In the following section, a more detailed account of research trends in each element of well-being messaging is presented. In particular, the prevalence of various element-specific aspects in different product types and across time is considered.

Well-being Messaging Elements

Product

Product was the second most studied well-being message element, after message, across the time span of this review, with recent time periods showing an upward trend in product-related research (**Figure 4**). A closer look at the overall picture (depicted in **Figure 8**), however, illustrated that inclusion of products other than milk, providing a point of comparison between products with regard to well-being messaging, was the main driver behind this increase in product-related research. Many other product-related aspects, however, have received less attention across time. For example, whether the product of interest indicated preserving animal welfare and how it might be associated with well-being messaging has mainly been investigated in the most recent time period (2016–2019). This was also the case for milk origin, shelf life or expiry date, and consumer experience. Studies of product-related attributes, such as price, size, sensory attributes, production or processing method, measured healthiness or nutritiousness, and nutrient contents, while being present in most of the time periods, have not played a key part in the increase in number of product-related studies in recent years. Instead, studies focused on packaging (material and design) and comparisons between plain and flavoured milk have increased between 2016 and 2019.

In addition, **Figure 8** illustrates a lack of product-related well-being messaging studies on product types other than plain milk (fluid and unspecified). Notably, plain milk powder had never been investigated in the empirical studies of the topic prior to this decade, and research on milkshake has mostly concerned nutrient content.

Message

Message, the most empirically studied well-being messaging element, was arguably the most time-dependent one too. A more detailed precis of message-related studies (**Figure 9**) showcased the rise and fall of well-being messages carrying various contents across the time. An example of this pattern would be r-BST-free messages (i.e., the cows have not been treated with the hormone recombinant bovine somatotropin). These messages, while not studied prior to 2001, have been the third most commonly empirically examined type of well-being message between 2001 and 2010 (i.e., 10 studies in total). Since 2011, however, only three studies have investigated r-BST-free messages. Another example would be well-being messages around safety or quality of the product that have only been studied between 2006 and 2015 (i.e., four times in total), with no appearance in the records prior to after this time period. On the other hand, well-being messaging on production or processing method, utilisation of artificial growth hormone, genetic modification, lactose, sugar, minerals content, warning messages, the use of front-of-pack (FOP) profiling systems, certified well-being messages, and attention to format and design of well-being messaging have all gained momentum lately. An exception to the time-dependent manner of message-related studies was certainly fat content, which has been an ever-present message across time.

With regard to product type, well-being messaging on sugar content has been, not surprisingly, more prevalent for flavoured milk. Flavoured milk was also a common product type of interest for investigations of FOP profiling systems and warning messages. Much like product-related research, plain milk powder had rarely been subjected to message-related research prior to 2010. The 13 studies published since then have included strengths, wording, format or design of well-being messaging, FOP profiling systems, disease/risk reduction, function, general health, general nutrition-related well-being messaging, fat, lactose, and general nutrients content, and production/processing method well-being messages.

Sender

Sender-related research, while very much lacking till 2006, showed considerable increase across several aspects for the subsequent 10 years. This increase in sender-related studies, however, has not continued in the past four years (**Figure 4**). Despite the decrease, various sender-related aspects have been investigated (**Figure 10**). Manufacturer image (e.g., years in business, familiarity to the consumer, and being known as an organic brand) has been frequently studied in relation to well-being messaging lately. Producing country, brand, endorsements or verifications, geographical span of a brand, and whether the product was locally produced or not have all been subjects of empirical enquiry. Manufacturer motivations or barriers to take certain approaches toward well-being messaging, as well as brand, however, were areas of research that have not received much attention across time. A dearth of prior empirical research on this topic was also noticeable with regard to product types other than plain milk (fluid or unspecified) and flavoured milk.

Receiver

Receiver-related research (**Figure 11**) quite expectedly included many instances of demographic comparisons (e.g., age, gender, education, and ethnicity). Through time, however, more nuanced receiver-related factors have been included in investigations. Particularly, in the past four years, receiver weight or Body Mass Index (BMI), health, knowledge of production or processing methods, knowledge of specific well-being messages, as well as comparing between various consumer segments or clusters, have each featured at least five times. On the contrary, other factors, such as diet or eating habits, healthy lifestyle, bodily measures, lactose intolerance, individual differences (traits, values, attitudes, or behaviours), food-related individual differences, beliefs and attitudes toward dairy products, sensory preferences, and shopping attitudes and behaviours, while being pivotal to knowing the receiver, have not been subjected to more than 3–10 studies each across the span of the review (i.e., 65

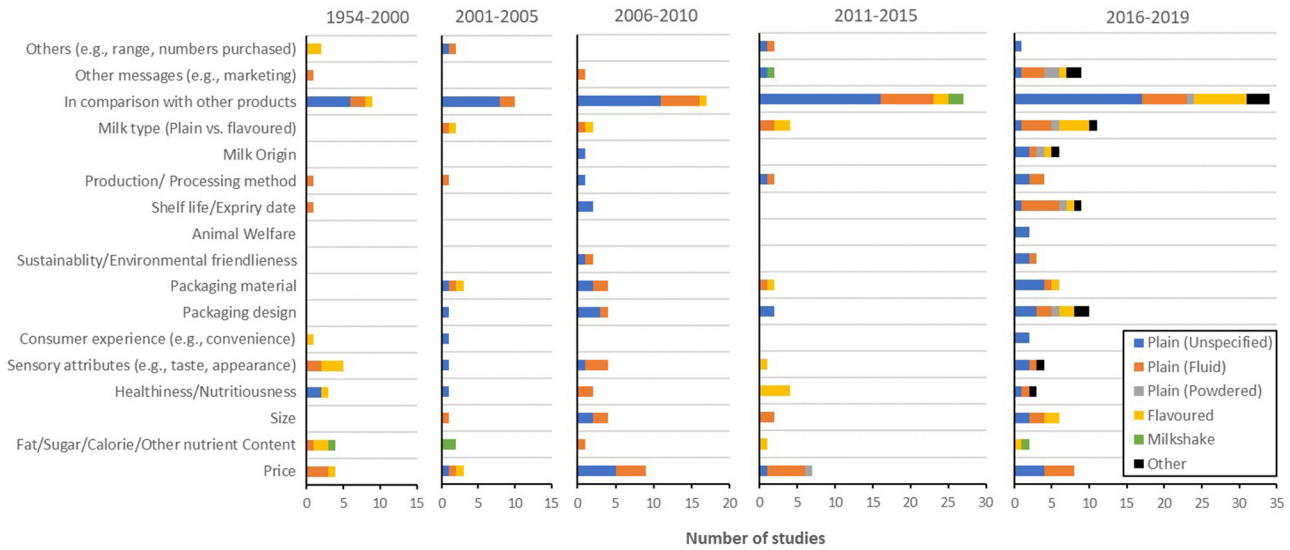

FIGURE 8 | Prevalence of product-related empirical studies for each milk type [i.e., plain (unspecified), plain (fluid), plain (powdered), flavoured milk, milkshake, and other milk drinks] during each period (i.e., 1954–2000, 2001–2005, 2006–2010, 2011–2015, and 2016–2019).

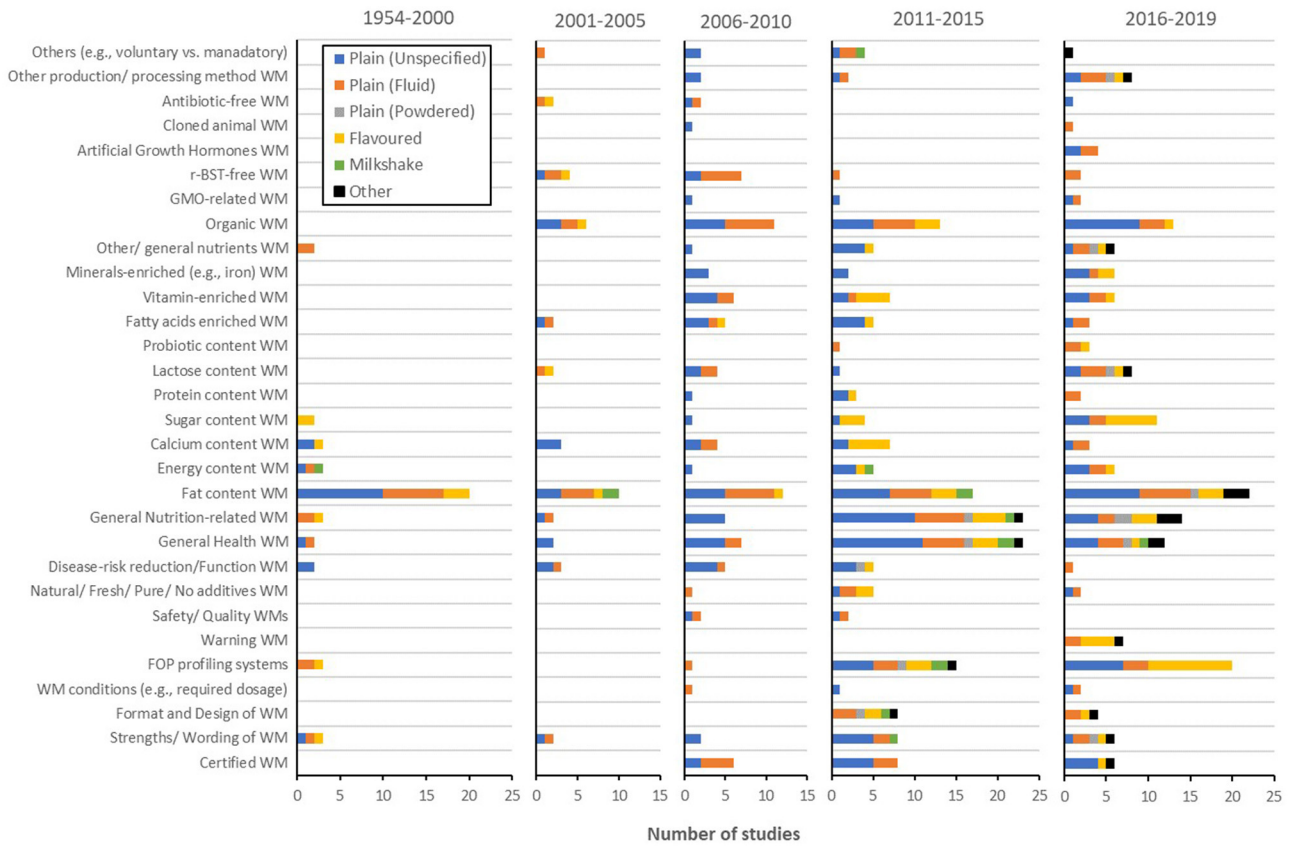

FIGURE 9 | Prevalence of message-related empirical studies for each milk type [i.e., plain (unspecified), plain (fluid), plain (powdered), flavoured milk, milkshake, and other milk drinks] during each period (i.e., 1954–2000, 2001–2005, 2006–2010, 2011–2015, and 2016–2019). WM, well-being messaging; FOP, front-of-pack; r-BST, recombinant bovine somatotropin; GMO, genetically modified.

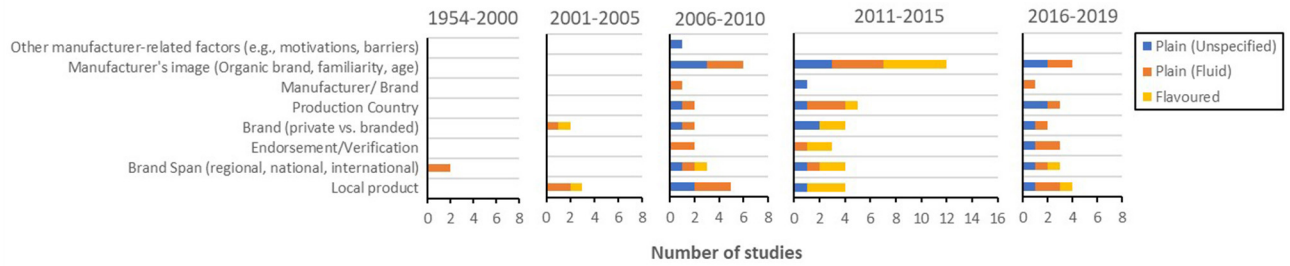

FIGURE 10 | Prevalence of sender-related empirical studies for each milk type [i.e., plain (unspecified), plain (fluid), plain (powdered), flavoured milk, milkshake, and other milk drinks] during each period (i.e., 1954–2000, 2001–2005, 2006–2010, 2011–2015, and 2016–2019).

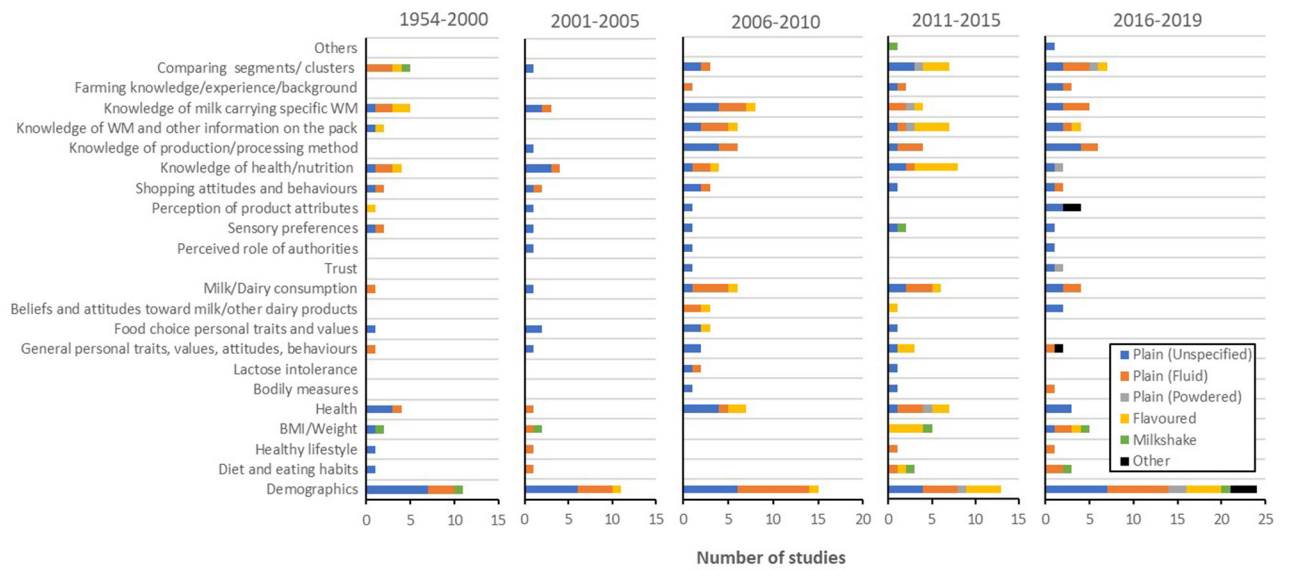

FIGURE 11 | Prevalence of receiver-related empirical studies for each milk type [i.e., plain (unspecified), plain (fluid), plain (powdered), flavoured milk, milkshake, and other milk drinks] during each period (i.e., 1954–2000, 2001–2005, 2006–2010, 2011–2015, and 2016–2019). WM, well-being messaging; BMI, body mass index.

years). Lack of studies on these factors has certainly not been addressed in the past few years. In addition, trust of a receiver in the messages relayed by milk, or a perceived role of authorities, has each only featured in three empirical studies to date.

A lack of studies investigating milk powder as the product of study was a common observation across all elements and was particularly evident here with only 10 studies across the two most recent time periods (2011–2105 and 2016–2019) combined. Comparing these two time periods, it seemed that flavoured milk has also experienced a significant drop in empirical interest lately.

Medium

Medium was the most clearly overlooked element of well-being messaging research; it has only been included in 27 studies across time, only one-sixth of message-related studies (**Figure 4**). A closer look at the past four years, however, was more alarming (**Figure 12**). Placement of well-being messaging on the package (FOP, BOP, or both), for instance, which was studied for various product types between 2011 and 2015, has not been studied at all in this period. Also, research into inclusion of well-being messaging in advertisements, point of choice delivery, and modes of delivery other than the food package has, it would seem, been ignored in recent years. Research on various delivery designs or formats (e.g., verbal, auditory, or imagery cues) and inclusion of marketing strategies in association with well-being messaging for milk, however, has increased lately.

As the number of medium-related studies was quite miniscule, further breaking it down by product type would not provide a meaningful point. Powdered milk, for example, has been studied one time and milkshake three times; then again, the other product types have not been subjected to many more medium-related studies.

Context

Context-related research in the literature included a variety of factors from point-of-choice-related factors to annual, seasonal, or monthly differences, living conditions, geographical location, and market regulations (**Figure 13**). While most of

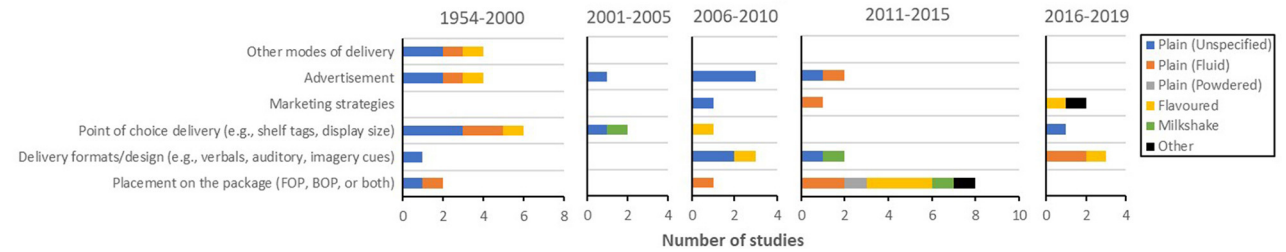

FIGURE 12 | Prevalence of medium-related empirical studies for each milk type [i.e., plain (unspecified), plain (fluid), plain (powdered), flavoured milk, milkshake, and other milk drinks] during each period (i.e., 1954–2000, 2001–2005, 2006–2010, 2011–2015, and 2016–2019). FOP, front-of-pack; BOP, back-of-pack.

these factors have received some attention throughout years, the association between well-being messaging and regulations, geographical location, living settings, point of choice factors, and discounts and/or promotions, among others, have been the main variables of interest in the past four years. In contrast, time, choice conditions, availability, and convenience have not been investigated lately. With regard to less-studied product types and context, whereas milk powder has been studied a few times recently, milkshake was only studied one time back in 2004.

Outcome

Well-being messaging for milk, being a multidisciplinary field of research, has attracted a wide range of outcome variables. **Figure 14** pictures this variety in association with the scholarly disciplines involved in milk well-being messaging research. Within Food, Agriculture, and Biological Sciences, for instance, price and/or value for money, willingness to pay or purchase, reaction to or use of well-being messaging, overall acceptability, choice, or preference, have each been studied at least 20 times. Interestingly, although, none of these were among the top two outcomes of interest in Nutrition and Dietetics, namely healthiness, safety, or nutritional quality, and prevalence of well-being messaging. Comparing more distant disciplines, the differences between target outcomes became even obvious. Whereas, sales-related outcomes (price and/or value for money, sales, demand, and/or market share, purchasing motivation, attitude, or behaviour, willingness to pay or purchase, and purchase volume, frequency, or expenditure) have been studied 45 times in Economics, Econometrics, and Finance, they have never been subjected to study in Communication. Furthermore, it was surprising to witness the absolute lack of studies targeting hedonic evaluation, overall liking, or sensory preference in Medicine, Public Health, and Health Professions and in Marketing, Management, and Accounting, or price and/or value for money, and sales, demand, and/or market share in Psychology.

DISCUSSION

An interdisciplinary scoping review of published records on well-being messaging for milk uncovered 246 records, which were analysed from several chronological, geographical, and methodological perspectives, as well as research discipline, product types, and well-being messaging elements of interest. Here, the key findings, strengths, and limitations of this research, and recommendations for future research are discussed.

Key findings

Eight key findings emerged through this review. First, while as many as 60 review studies were identified in the existing literature, it was surprising to find that milk well-being messaging, *per se*, has not been the main subject of any scoping review to date (see **Supplementary Material**), and this is despite the considerable number of empirical and nonempirical records that have been published on the topic. Also, given that milk well-being research cuts across various scientific disciplines, a lack of an interdisciplinary approach to reviewing the literature on well-being messaging for milk seems to have played a role in clear discipline-specific knowledge gaps, which are further discussed in this section. Altogether, it is evident that milk well-being research, in its seventh decade of existence, is very much missing scoping reviews aimed at discovering the boundaries of research in the field, clarifying key terms and definitions, and identifying gaps through mapping the literature. This research thus provides a stepping-stone to rectify this limitation, paves the way for more specific research enquiries to bridge the identified gaps, and invites multidisciplinary efforts in milk well-being messaging research.

Second, given that well-being messaging happens in an environment where optimal design, legal compliance, and effective delivery are keys to success, limited research published in disciplines, such as Law or Communication, especially in the past decade, or absolute lack thereof in Arts, Design, or Human Factors and Ergonomics was unexpected observations. Absence of research in these fields may be responsible for largely unheeded well-being messaging elements, such as medium or context. Furthermore, whereas Marketing, Management, and Accounting have published over 30 studies on well-being messaging for milk through the review time span, only three studies have, to date, entertained the sender of well-being messaging. This suggests that future undertakings within the discipline, particularly in areas, such as Organisation Science, Leadership, Process, Innovation, or Strategic Management, are the keys to offer an understanding of the processes of decision-making, design, implementation, and delivery of well-being messaging for milk in organisations.

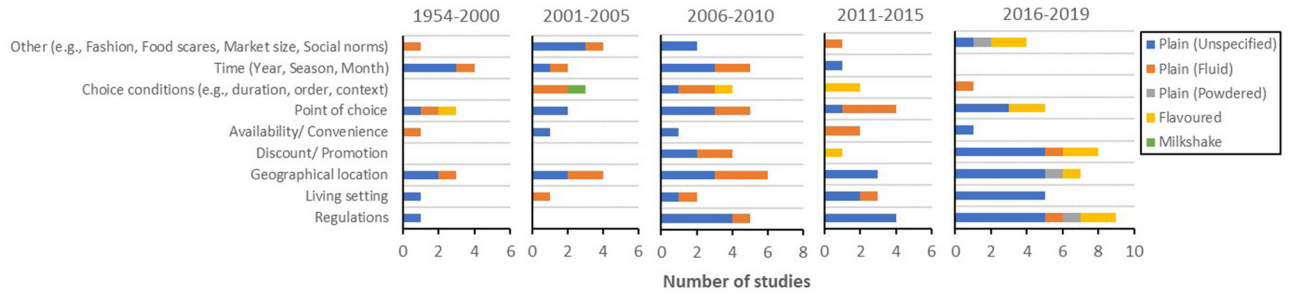

FIGURE 13 | Prevalence of context-related empirical studies for each milk type [i.e., plain (unspecified), plain (fluid), plain (powdered), flavoured milk, milkshake, and other milk drinks] during each period (i.e., 1954–2000, 2001–2005, 2006–2010, 2011–2015), and 2016–2019.

Third, regarding geographical regions, this review revealed that East Asia, including China and India, nearly half of the World's population, have never been empirically studied before 2006 and have only been subjected to 13 enquiries since then. This is more interesting when considered in conjunction with the number of empirical research studies conducted in Oceania to date ($N = 22$), with a population <45 million. A closer look at the elements of well-being messaging studied across these geographical regions, however, adds further insight. Interestingly, while receiver has been studied in 69.2% of studies carried out in the East Asia region, sender has been investigated in 59.1% of studies conducted in the Oceania region; these are the highest percentages of receiver and sender studies (of total) among all regions. Additionally, when compared with other regions, context in East Asia (53.8% of total studies) and product in Oceania (90.9% of total studies) have been of specific empirical interest. Hence, while East Asia has received meagre studies on milk well-being messaging in general, East Asian receivers and context have been of particular interest. On the other hand, research in Oceania has paid specific attention to the product and the senders of milk well-being messaging. Hence, it might be the case that these findings reflect upon the fact that milk well-being messaging research in East Asia, a major importer of milk (40), and Oceania, a major exporter of milk (40), has responded to the trade market needs for effective well-being messaging *via*: (a) providing a better understanding of consumers and market-related factors in East Asia, and (b) inspecting product- and producer-related aspects in Oceania. Whether scholarly research is ultimately missioned to follow market realities or to provide a comprehensive regime of research investigating all elements of well-being messaging at the same level, however, is a question to be considered collectively by academic society and the dairy industry.

Fourth, milk powder was a subject of only five out of 177 empirical studies of well-being messaging for milk. This figure seems very small when compared to studies of fluid plain milk (55 studies). Considering that most of the 91 studies featuring plain milk of unspecified format are likely to refer to fluid plain milk, the focus on milk powder in plain milk well-being messaging research appears even smaller. Hence, the ratio of well-being messaging research for milk powder to that of plain milk, regardless of its format, is about 1–30. According to Mintel Global New Product Database (41), nearly 1 in 5 plain milks launched in 86 markets around the globe between 2016 and 2019 was milk powder (1,324 out of 6,805 products). In China, for example, 247 of 1,005 plain milks launched in this period were milk powders (24.5%; 39), and, remarkably, none of the five studies that included milk powder in this review were conducted in China. Therefore, there is no doubt that there is a gap in research for well-being messaging concerning powder milk and more so in certain markets.

Fifth, while empirical studies of well-being messaging for milk have been ever-increasing, studies on medium and sender are two elements that have not followed this trend. In fact, the past four years (2016–2019) have shown decreased numbers of empirical studies on medium and sender compared with the two previous periods. While this, as discussed above, might be due to lack of sufficient research in certain disciplines, it might also point to the complexity of conducting research on these elements. Cutting edge research on medium-related (and context-related) aspects of well-being messaging is sometimes reliant on employing novel data collection techniques such as eye-tracking to investigate spatial cognition of product package (42–44) and/or virtual reality to assess consumer interaction with various package designs or point of choice settings (45–47). These methods, while being widespread in other fields of research (48–52), need to become more commonplace in milk well-being messaging research [see (53), for an exception]. Likewise, studies engaging with dairy production, distribution, and retailing functions to understand their motivations, barriers, and procedures regarding design, implementation, and evaluation of well-being messaging [e.g., (54)], despite being more challenging than convenience sampling or collecting on-the-pack information, are vital to sender-related research. Employing lessons learnt from organisation research on other topics such as new product development (52, 55, 56) or food packaging (57) can be helpful.

Sixth, more commonly studied elements, namely message, product, and receiver, have all witnessed consistent increase in scholarly attention during the past two decades. The increase in number of studies, however, does not necessarily mean that our understanding of aspects of well-being messaging related to these elements is also growing comprehensively. Research on product, for instance, has disproportionally involved the inclusion of food products other than milk as points of comparison. Many other important product-related aspects,

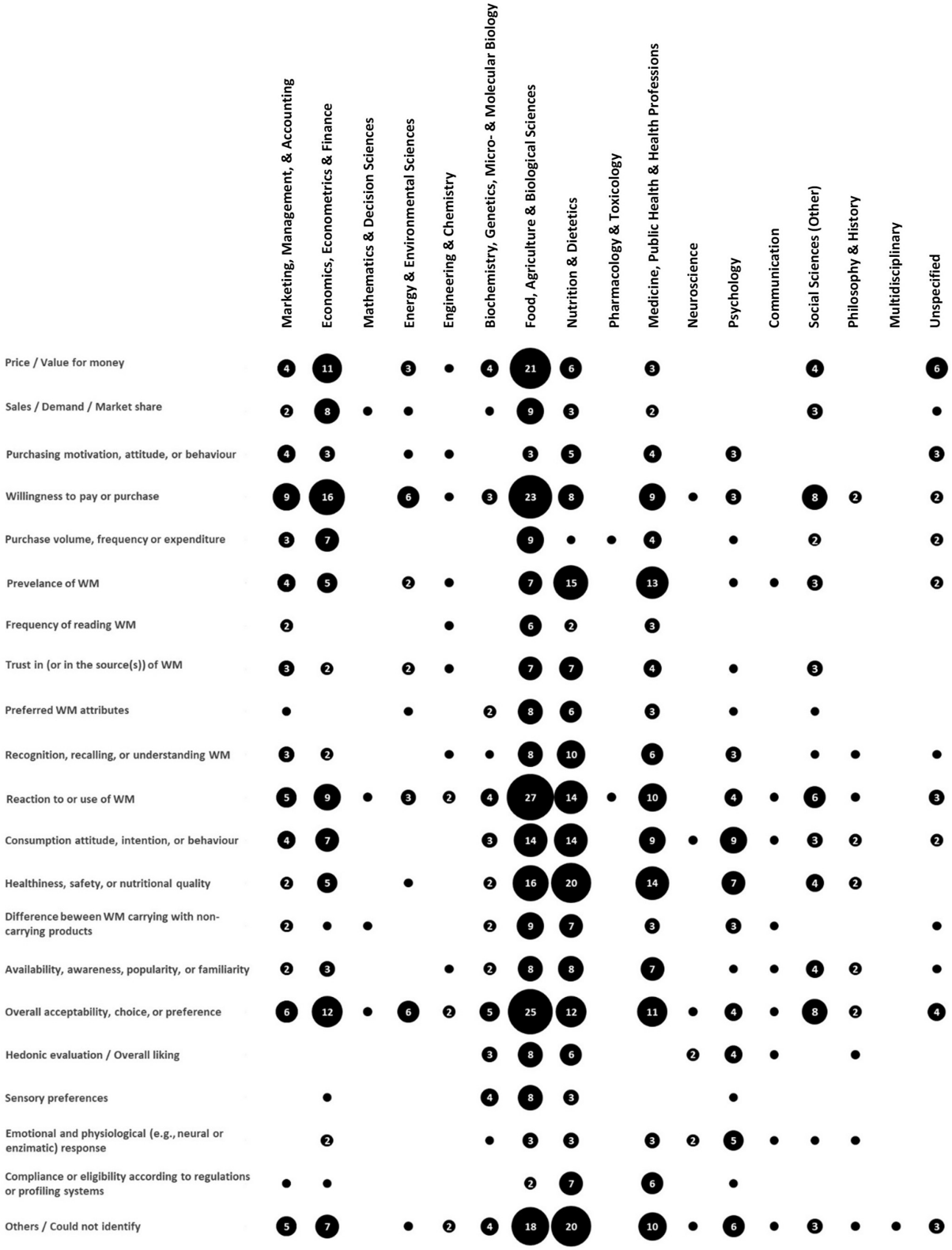

FIGURE 14 | Prevalence of particular outcomes of empirical interest in each discipline. Numbers in bubbles specify the prevalence. Note that bubbles with no number represent only one record.

such as milk origin, shelf life, packaging (material and design), sensory attributes, consumer experience, size, production or processing method, measured healthiness or nutritiousness, and nutrient content, however, have arguably received insufficient attention across time. This is also the case for receiver-related research, where inclusion of demographic information (e.g., age, gender, education, and ethnicity) has been and increasingly has become the most common practise. This is despite the existence of many understudied aspects, such as consumer diet, eating habits, healthy lifestyle, bodily measures, lactose intolerance, general and food-related individual differences (traits, values, attitudes, or behaviours), beliefs and attitudes toward dairy products, sensory preferences, and shopping attitudes and behaviours that are crucial pieces of the puzzle. In regard to message-related research, however, at least, during the past four years (2016–2019), various message types, formats, designs, and content have all been subjected to empirical research.

Seventh, a closer look at message-related research reveals a time-dependent pattern in empirical studies of particular well-being messages for milk. As such, antibiotic-free or r-BST-free messages or well-being messages around safety or quality of the product were all more common between 2001 and 2015, compared with other times periods. This might be explained through short-lived food trends or food scares during this period. A search in Google Trends archival data (performed on 9th March 2021), for instance, shows that worldwide Google searches were substantially more common for the terms "rBST in milk" prior to 2010 and "antibiotic in milk" prior to 2008, compared with years after. This showcases a significant increase in public attention to these topics, which might have been triggered by other regional or global events at the time, possibly convincing the dairy industry to consider relevant well-being messaging, and ultimately encouraging higher scientific attention to the topic. The infamous case of melamine adulteration in milk and infant formula in 2008, which resulted in death and hospitalisation of babies and adults across China (58), for instance, caused a peak in scholarly interest in safety and quality well-being messaging for a short period thereafter. Whether other emerging trends, such as messages on production/processing method, such as cloned animals, artificial growth hormones, genetically modified, and organic, or messages on lactose, probiotic, vitamins, and minerals content, will stand the test of time, however, needs to be reassessed in future reviews of this topic. Having said that, there are aspects, including fat, calcium, and sugar content; strengths and wording of well-being messages; and well-being messaging on health and nutrition values of milk, that have generally remained mainstream in message-related research.

Eighth, whereas wide-ranging outcome variables have been of interest in milk well-being messaging research, this research highlights that various disciplines have focussed on specific outcomes and, sometimes, to the extent that they have missed other important ones. A lack of interdisciplinary reviews of the topic has not helped this situation either. Discipline-specific tunnel vision is clear in several areas, for example, lack of consideration of price, value for money, sales, demand, and market share in research published in Psychology or Communication disciplines; or the omission of hedonic evaluation, overall liking, and sensory preference factors in Medicine, Public Health, and Health Professions discipline, and in Marketing, Management, and Accounting research. Hence, taking an interdisciplinary lens would result in a more complete depiction of milk well-being messaging, one that is more capable of guiding optimal well-being messaging policies and practises.

Strengths and Limitations

The research was crucially founded on three proposed shifts in well-being messaging research paradigms. These, on their own, can be considered as important contributions to the topic. First is the use of "well-being," as a multifaceted concept covering various biopsychosocial domains instead of "health," which is commonly understood as the state of being physically healthy. Also, "well-being messaging" has been suggested as an umbrella term that contains various types, frames, designs, contents, and strengths of well-being-related food labels appearing on- and/or off-package. Adopting these terms and definitions was the key to bringing together a seemingly diverging literature around what is essentially *well-being messaging*. Second, well-being messaging was modelled as an act of communication and, hence, consisted of distinct communication elements. This novel approach allowed the identification of aspects of milk well-being messaging that have not received much attention in the past, e.g., sender and medium. Third, well-being messaging, particularly when broadly defined, lends itself to various research enquiries that, in many cases, cuts across various scientific disciplines. Taking an interdisciplinary approach to reviewing milk well-being messaging research showcased discipline-specific vision that caused some disciplines to focus on specific outcomes while missing important ones. Following this approach in future scholarly research will provide more comprehensive and informative research on milk well-being messaging.

However, there are also a few limitations to note. First, given the overwhelming number of identified records, and in line with the recommendations for conducting scoping reviews, quality appraisal was not performed on identified records. Second, in order to maintain the feasibility of this scoping review, eligibility criteria were used to philtre out records with no access to full texts through institutional holdings available to the authors, potential grey literature indexed in other data sources that also did not appear in cited in-text or in reference lists of the general well-being messaging database records, non-English records, and records in which findings exclusive to milk or other product types of interest were not separated from other products (e.g., dairy products, milk, and plant-based milk substitutes). Third, while taking an interdisciplinary approach to conducting this scoping review allowed an open approach to emerging themes and to accommodate wide-ranging designs, variables, and outcomes of interest, this approach also limited the possibility for the fine-grained data synthesis a systematic review with a narrower aperture would allow.

CONCLUSION

This research is the first interdisciplinary scoping review of well-being messaging for milk. It features unique aspects, including: (i) offering an all-embracing definition of well-being messaging; (ii) suggesting a framing model for well-being messaging research; (iii) taking an interdisciplinary approach to the search, synthesis, and analysis of literature; (iv) inclusion of both empirical and non-empirical records; (v) providing a comprehensive overview of milk well-being messaging research (across scientific disciplines, time, geographical locations, product type, and research methods) as well as a closer look at particular knowledge gaps that require further attention. Most importantly, existing non-empirical literature to date has not included an exclusive review of research on well-being messaging for milk. The results of this review also highlight notable knowledge gaps in the empirical literature, particularly with regard to milk well-being messaging research in geographical locations other than North America, West Europe, and Oceania, on product types other than fluid plain milk, and on messaging elements other than product and message. Insights from this research not only warrant the need for further academic undertakings in certain domains of milk well-being messaging but also inform practitioners and policy-makers who can understand chronological well-being needs and concerns of milk consumers in various markets as reflected in areas of research focus across time.

AUTHOR CONTRIBUTIONS

SM and JH designed the study, screened the results, and wrote the paper. SM conducted the literature search and analysed and charted the results. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.688739/full#supplementary-material

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