DIETARY CARBOHYDRATE DIGESTIBILITY AND METABOLIC EFFECTS IN HUMAN HEALTH

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PUBLISHED IN: Frontiers in Nutrition and Frontiers in Microbiology

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88963-231-2
DOI 10.3389/978-2-88963-231-2

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DIETARY CARBOHYDRATE DIGESTIBILITY AND METABOLIC EFFECTS IN HUMAN HEALTH

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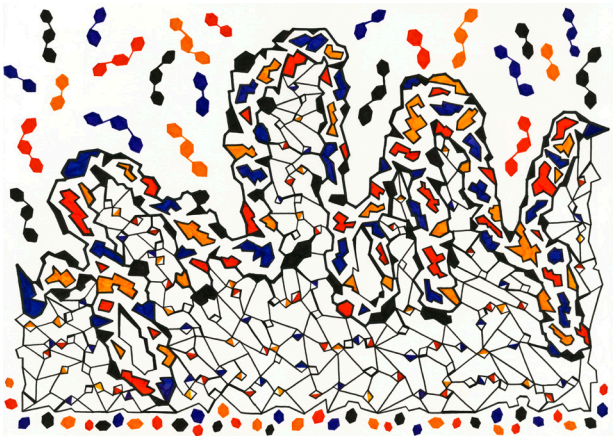

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Citation: Moreno, F. J., Hernandez-Hernandez, O., Rastall, R., eds. (2019). Dietary Carbohydrate Digestibility and Metabolic Effects in Human Health. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88963-231-2

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frontiers in Nutrition

EDITORIAL
published: 15 October 2019
doi: 10.3389/fnut.2019.00164

Editorial: Dietary Carbohydrate Digestibility and Metabolic Effects in Human Health

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Keywords: prebiotic, dietary fiber, gut microbiota, bovine milk oligosaccharides, human milk oligosaccharides, enzymatic glycosylation, non-digestible carbohydrates, cross feeding

Editorial on the Research Topic

Dietary Carbohydrate Digestibility and Metabolic Effects in Human Health

The links between some carbohydrate components of the human diet and health have been understood for decades. Beyond the provision of energy, digestible carbohydrates can have other impacts on host health including acting as dietary fiber and prebiotics. In order to qualify as either of these, carbohydrates need to be largely indigestible by the human gut (1). As discussed by Hernandez-Hernandez et al., current ways to measure the digestibility of carbohydrates *in vitro* remain challenging and are very poorly developed in relation to our ability to determine their impact on health in human trials. The most commonly used "*in vitro* digestion" methods (based on the use of microbial amylolytic enzymes combined with invertases) do not model the complex degradation of carbohydrate structures in the gut with any accuracy and these authors point the way toward using mammalian brush border enzyme extracts or, in the future, cloned mammalian enzymes (2).

One challenge in research of non-digestible carbohydrates and gut health is a lack of understanding of the functional ecology of the gut. At present, developments of novel non-digestible carbohydrates take place with a very limited knowledge of how they will be handled by the gut microbiome. Degradation and metabolism of dietary carbohydrates involve a complex multispecies action followed by metabolic cross feeding on the products. Currently, little is understood of the cross feeding networks that occur in the gut. In their review, Crost et al. reported on some of the details of cross feeding relationships involving starch degrading and mucin degrading *Ruminococcus* species. As degradation of carbohydrates involves bacterial glycoside hydrolases, further investigation of this interrelationship is needed. Study of the glycosyl hydrolases, expressed by specific gut bacteria under nutritional regimes is very challenging and will require the development of new techniques in the future. Blanco et al. discuss the application of *in silico* techniques to characterize the glycosidic capacity of a potentially beneficial bacterium, *Faecalibacterium prausnitzii*, potentially facilitating the design and manufacture of prebiotics targeted at specific organisms.

There are several prebiotic oligosaccharides on the market and the market leaders are the fructo-oligosaccharides and galacto-oligosaccharides, manufactured using enzymatic approaches. A review by Martins et al. reported the potential promise for the development of novel bioactive structures, and future alternate sources of nutritional carbohydrates that could be developed in future. This theme is expanded upon by Garcia-Valle et al. who evaluated the use of unripe plantain flour as a functional ingredient. Enzymatic glycosylation provides the potential to bring about synthesis of many novel bioactive molecules, such as novel prebiotic carbohydrates and derivatives of other nutritional compounds

OPEN ACCESS

Edited and reviewed by:
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Specialty section:
This article was submitted to Nutrition and Food Science Technology, a section of the journal Frontiers in Nutrition

Received: *17 June 2019*
Accepted: *01 October 2019*
Published: *15 October 2019*

Citation:
Rastall RA, Moreno FJ and Hernandez-Hernandez O (2019) Editorial: Dietary Carbohydrate Digestibility and Metabolic Effects in Human Health. Front. Nutr. 6:164. doi: 10.3389/fnut.2019.00164

with improved functionality. An example of this is given by Gonzalez-Alfonso et al. who have used the enzyme cyclodextrin glucosyl transferase to α-glucosylate (-)-epigalocatechin gallate, to improve pH-, thermal stability, and antioxidant activity. Glycosylation of plant polyphenols may improve their activity and bioavailability more generically (3).

It is well-known that human milk contains an extremely complex array of oligosaccharide structures and these are believed to play an important role in infant nutrition, acting as prebiotic carbohydrates, anti-adhesive agents inhibiting the adhesion of pathogens and also acting as cell signaling molecules (4). It is, however, very difficult to carry out any kind of mechanistic study in infants and animal models are needed. Rudloff et al. describe the use of a pig model of necrotizing enterocolitis to study the metabolism of human milk oligosaccharides (HMO). They have used mixtures of oligosaccharides to study metabolism in the colon and excretion in urine. Demonstration that HMO can be absorbed, at least to some degree, from the human gut helps to explain some of the biological activities ascribed to HMO.

Despite the health benefits of HMO, it is not a trivial task to manufacture these oligosaccharides on a commercial scale. Only two human milk oligosaccharides have currently been put into large scale commercial production (i.e., 2′-fucosyllactose and lacto-N-neotetraose), although a number of new HMO products are in the commercial pipeline (5). Other non-digestible carbohydrates, however can mimic some of the effects of HMO (Verkhnyatskaya et al.) and galacto-oligosaccharides and fructo-oligosaccharides are both now added to infant formula. Verkhnyatskaya et al. explore the potential of other non-digestible carbohydrates to provide nutritional functionality in infant formulae in the future.

An alternative approach to the isolation or synthesis of human milk oligosaccharides might be to source complex oligosaccharides from bovine milk. As discussed by Robinson, these oligosaccharides are present in lower quantities than in human milk and the bovine oligosaccharides do not have exactly the same structures. They may, however, be more amenable to large scale extraction and they are being studied for their bioactivities and nutritional potential. For instance, Kuntz et al. describe the effect of bovine milk oligosaccharides from different cattle breeds on the growth of mammalian cells *in vitro*. The oligosaccharides had a dose-dependent growth inhibition effect on HT-29, Caco-2 and non-transformed human intestinal cells. They also induced differentiation in the non-transformed intestinal cells.

It is clear from the papers in this Research Topic that the field of non-digestible carbohydrates has many avenues to explore. The impact of such carbohydrate molecules on the gut microbiome is currently an area of intense investigation and hopefully will lead a detailed understanding of how specific carbohydrate structures can influence the ecology of the gut to produce specific metabolic signatures and how these can then impact on human health and wellbeing. It is becoming clear that dietary carbohydrates can also, at least *in vitro*, impact upon mammalian cell physiology. At the present time the extent and nature of metabolic cross feeding in the gut, the impact of microbial metabolites on human health and the degree to which direct interactions with cellular physiology occur *in vivo* in healthy humans are far from clear. However, pioneering *in vitro* approaches such as the culture of human gut microbiota in an anaerobic intestine-on-a-chip may serve as a cutting edge tool for mimicking the host-microorganism interactions in the human intestine (6, 7).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work has been financed by the Spanish Ministry of Economy, Industry and Competitiveness (Project AGL2017-84614-C2-1-R) and Science, Innovation and Universities (Project RTI2018-101273-J-I00).

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Conflict of Interest: The 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.

frontiers in Nutrition

OPINION
published: 07 May 2019
doi: 10.3389/fnut.2019.00061

In vitro Digestibility of Dietary Carbohydrates: Toward a Standardized Methodology Beyond Amylolytic and Microbial Enzymes

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Keywords: non-digestible carbohydrates, slowly digestible carbohydrates, brush border enzymes, carbohydrases, non-starch oligosaccharides, small intestinal brush border membrane vesicles

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Nutrition and Food Science Technology, a section of the journal Frontiers in Nutrition

Received: *25 February 2019*
Accepted: *17 April 2019*
Published: *07 May 2019*

Citation:
Hernandez-Hernandez O, Olano A, Rastall RA and Moreno FJ (2019) In vitro Digestibility of Dietary Carbohydrates: Toward a Standardized Methodology Beyond Amylolytic and Microbial Enzymes. Front. Nutr. 6:61. doi: 10.3389/fnut.2019.00061

BACKGROUND AND LIMITATIONS OF THE CONVENTIONAL *IN VITRO* METHODS TO ASSESS DIGESTIBILITY OF DIETARY CARBOHYDRATES

Scientific evidence of human digestion of foods gathered during the last decades has strengthened the relationship between digestibility and the possible effects of foods on human health (1). The high complexity of the gastrointestinal (GI) environment and the lack of an easy direct access to most of the parts of the GI tract (2) with the exception of the oral cavity, prevent the broad implementation of *in vivo* animal, or human trials for assessing the digestion, absorption, and metabolism of dietary food ingredients. Therefore, *in vitro* testing has a central place in the current investigations of food's digestive fate.

Non- or slowly digestible carbohydrates may have appealing properties that are associated with a series of beneficial physiological effects, such as low-calorie (important in preventing obesity), low-glycemic (helpful in managing diabetes and cardiovascular disease), and low-digestible (helpful in reducing the intestinal transit time and in positively modulating the gut microbiota composition and activity). These beneficial effects have sparked the interest, from both academic and industry perspectives, in carbohydrates showing resistance to digestion (or slow digestibility) and absorption in the small intestine and thus being available in the large intestine as substrates for fermentation by gut microbiota.

Since Southgate published, in the late 1960s, two methods for measuring available (3) and unavailable (4) carbohydrates in foods by hydrolyzing starch with amyloglucosidase and pullulanase of fungal origin, a plethora of models ensued with the common rationale of using amylolytic enzymes that, in some cases, may be combined with a microbial invertase (5). Remarkably, the substrate specificity, hydrolytic mechanism and influence in physiologic responses of amylolytic enzymes from fungal or bacterial sources commonly used in *in vitro* starch digestion assays has been demonstrated to be noticeably different from their equivalent in mammals (6, 7).

The current state-of-the-art reveals that human gastrointestinal digestion of dietary carbohydrates is a multistage process, involving up to six different carbohydrases produced by three different organs (**Table 1**). The main function of the oral phase is the conversion of food into a homogenous mass during mastication, whilst the starchy carbohydrates undergo a very limited salivary α-amylolysis. The main site for carbohydrate digestion is the small intestine, where the chyme is mixed with α-amylase containing secretions of the pancreas. In addition, the key role

played by the mucosal disaccharidases embedded in the small intestinal brush border membrane vesicles that are responsible for the final stage of luminal digestion prior to absorption needs to be recognized (11, 12) (**Table 1**). Strikingly, the inclusion of small intestine mucosal carbohydrases has been ignored in the vast majority of *in vitro* models developed to date. This issue is particularly critical in the assessment of not only non-starchy carbohydrates, but also in the quality of starch digestion since the small intestine mucosal α-glucosidases have been reported to play an important role by influencing the rate, location and extent of glucose release which is associated with high glycaemia-related health issues such as diabetes and other metabolic syndromes (7). Nevertheless, recent research is addressing this caveat by developing alternative *in vitro* methodology aiming to better model the interaction of the carbohydrate with small intestine mucosal carbohydrases as explained below.

DEVELOPMENT OF ALTERNATIVE *IN VITRO* MODELS TO ASSESS CARBOHYDRATE DIGESTIBILITY USING MAMMALIAN SMALL INTESTINAL BRUSH BORDER CARBOHYDRASES

TIMcarbo: A Dynamic GI Tract Model Mimicking the *in vivo* Physical Processing and Temporal Changes in Luminal Conditions

This method has been built on the well-known TNO Gastro-Intestinal Model (TIM), which is a multi-compartmental dynamic model that was developed by TNO in The Netherlands in the early 1990s in response to industrial demand to study food products under more physiologically relevant conditions as compared to contemporary digestion models (13, 14). The TIMcarbo model includes an oral digestion phase by using a model of artificial mastication with a bacterial salivary α-amylase. After the luminal gastrointestinal digestion, which is simulated by adding pancreatic juice containing proteases, lipases and amylase, among other factors, the TIMcarbo mimics the carbohydrate uptake by the epithelium. This is achieved by dialysis of the products of digestion by a commercial rat brush border enzyme extract complemented with a bacterial β-galactosidase (15). The use of a microbial lactase is justified by the fact that the rat brush border extract contains a much lower activity than that found in humans. Finally, the *in vitro* methodology is combined with an *in silico* kinetic modeling that allows the prediction of glycemic response curves in humans.

This model is highly relevant to studying the digestion of whole foods and meals where the physical condition of the digesta changes over time, e.g., viscosity or particle size reduction that are key determinants of the rate of carbohydrate digestion and absorption *in vivo* (5, 16, 17). However, this refined method is not so suitable for screening studies or for testing the digestibility of carbohydrates available at low amounts, which is usually a limiting factor when the digestibility of novel carbohydrates produced at laboratory scale needs to be assessed.

Batch *in vitro* Digestion Assays Using Rat or Pig Small Intestine Mucosal Carbohydrases

Batch models do not pretend to mimic the physical and mechanical processes that occur *in vivo*. Consequently, in this type of digestion model the products of digestion are not removed and therefore, they do not model the absorption process. However, this type of model is especially suited for assessing the digestibility of isolated carbohydrates or single foods and can provide valuable information on mechanistic studies.

In line with the brush border enzyme digestion included in the TIMCarbo model, the use of rat small intestinal extract has been successfully applied for the assessment of digestibility of several prebiotic carbohydrates. These are non-digestible carbohydrates with the ability to selectively modulate the composition and/or activity of the gut microbiota, thus conferring benefit(s) upon host health (18), such as fructooligosaccharides (19), galactooligosaccharides (20, 21), or isomaltooligosaccharides (22).

In addition to the use of the rat intestinal extract, Lee et al. (23) investigated the contribution of the four main individual recombinant mucosal α-glucosidases (that is, glucoamylase, maltase, sucrase and isomaltase) on a range of unusual α-linked glycemic disaccharides with two glucose units. These authors provided intriguing differences in hydrolytic properties of the individual rat mucosal α-glucosidases to disaccharide substrates differing in glycosidic linkages. Furthermore, they concluded that mammalian mucosal carbohydrases must be used in *in vitro* assessment of digestion of glycemic carbohydrates instead of microbial digestive enzymes. More recently, a newly developed *in vitro* digestion model, based on the use of a commercial rat small intestinal extract under physiological conditions of temperature and pH, was successful for evaluating the digestibility of dietary oligosaccharides of degree of polymerization up to four with a requirement for a minimum of 0.5 mg of carbohydrate (24). This method demonstrated its capacity to distinguish between well-recognized digestible and non-digestible carbohydrates; the tested digestible carbohydrates were readily hydrolyzed whereas the oligosaccharides classified as non-digestible were barely hydrolyzed. Remarkably, a good correlation was found between this *in vitro* method and *in vivo* data collected on growing (25) or neonatal (26) rats and from aspiration of the gut content at the terminal ileum of healthy humans (27) on galactooligosaccharides and fructooligosaccharides digestion.

The high physiological and anatomical similarity of the pig and human digestive tracts (28) may provide additional advantages of using pig small intestinal material instead of that from rats. Thus, alternative methods based on the use of mammalian intestinal enzymes derived from pigs (6) or weaning piglets (29) have recently been proposed. Moreover, Tanabe et al. (30) successfully applied an improved AOAC 2009.01 method by using porcine small intestinal enzyme instead of fungal amyloglucosidase to accurately determine non-digestible oligosaccharides in marketed food products. In this

TABLE 1 | Human carbohydrases involved in dietary carbohydrate digestion.

Digestive carbohydrases	Type of enzyme	Glycoside hydrolase family[a]	Production organ/main site of digestion	Glycosidic linkage specificity	Main substrates[b]	Main products[b]
Salivary α-amylase[c]	Secreted (α-glucosidase)	13	Salivary gland/mouth	Glcα(1→ 4)Glc	Starch; linear maltooligosaccharides ($n > 6$)	Maltose; maltotriose; α-dextrins
Pancreatic α-amylase[c]	Secreted (α-glucosidase)	13	Pancreas/Small intestine	Glcα(1→ 4)Glc	Starch; linear maltooligosaccharides ($n > 6$)	Maltose; maltotriose; α-dextrins
Sucrase-isomaltase	Mucosal (α-glucosidase)	31	Small intestine (brush border membrane)/Small intestine	Glcα(1⇔2)βFru Glcα(1→ 4)Glc Glcα(1→ 6)Glc	Sucrose; isomaltose; maltose; maltotriose; α-dextrins	Glucose; fructose
Maltase-glucoamylase	Mucosal (α-glucosidase)	31	Small intestine (brush border membrane)/Small intestine	Glcα(1→ 4)Glc Glcα(1→ 6)Glc	Linear and branched maltooligosaccharides ($n = 2$–9)	Glucose
Lactase-phlorizin hydrolase	Mucosal (β-glycosidase)	1	Small intestine (brush border membrane)/Small intestine	Glcβ(1→ 4)Gal Glcβ(1→ 4)Glc	Lactose, cellobiose, cellotriose, cellulose	Glucose; galactose
Trehalase	Mucosal (α-glucosidase)	37	Small intestine (brush border membrane)/Small intestine	Glcα(1⇔1)αGlc	Trehalose	Glucose

[a] *According to CAZy database (http://www.cazy.org/) (8).*
[b] *Based on and updated from Alpers (9).*
[c] *Human salivary and pancreatic α-amylases have 94% amino acid identity although they are encoded by different genes (10).*

context, the use of small intestinal brush border membrane vesicles conveniently isolated and purified from post-weaned pigs has recently shown to be a reliable and straightforward strategy to evaluate prebiotic carbohydrate digestibility, under physiological conditions of pH, temperature and time. These prebiotics included lactulose, different mixtures of commercial galactooligosaccharides, and an emerging prebiotic as novel galactooligosaccharides derived from lactulose (31). Recently, a pioneering approach, based on the study of the trans-β-galactosylation activity of the pig β-galactosidase embedded in the brush border membrane vesicles, has provided significant insights into the reaction mechanisms involved in the human digestion of dietary carbohydrates and has informed the development of non- or slowly-digestible carbohydrates (32). Lastly, a novel optimized *in vivo* and *in vitro* ileal fermentation assay, based on growing pigs as an animal model for simulating digestion in human adults, has been reported last year (33). Interestingly, the predicted values for ileal organic matter digestibility (calculated on the basis of the *in vivo*/*in vitro* ileal fermentation) were quite similar to the values measured *in vivo*, warranting the potential use of appropriate, and refined *in vitro* approaches using intestinal material from growing pigs to replace *in vivo* studies, in relation to ileal digestibility.

THE NEED FOR HARMONIZATION AND STANDARDIZATION OF AN *IN VITRO* TEST METHODOLOGY FOR ASSESSING DIGESTIBILITY OF DIETARY CARBOHYDRATES

Despite recent advances in developing *in vitro* methods to assess carbohydrate digestibility, there is an obvious need to design a standardized batch gastrointestinal digestion method based on physiologically relevant conditions. By considering the state-of-art, it is clear that the mucosal disaccharidases embedded in the small intestinal brush border membrane vesicles must be considered in addition to α-amylases, since they play a key role in the digestion of carbohydrates and in the subsequent uptake of monosaccharides by the intestinal mucosa (**Table 1**). Additionally, the use of mammalian digestive enzymes should be prioritized over the use of microbial enzymes since the former better reflect the carbohydrase activities of enzymes of the human gastrointestinal tract. However, there are still important uncertainties that prevent the development of a standardized methodology in the short-term. These include (but not exclusively) the following aspects:

(i) A large variety of enzymes from different sources have been used to date, also differing in the assays used to determine the enzyme activity. This clearly impairs an appropriate characterization of the carbohydrases used as well as the comparison of outcomes from different studies.
(ii) The regular supply of commercial pig mucosal small intestinal enzymes is nonexistent whilst that of rats is restricted.
(iii) The expression of mucosal carbohydrases greatly varies among the different segments of the small intestine and is also driven by the type of diet, as well as the host genetics, health status and age, among other factors (34).

Possible solutions to overcome these drawbacks could be the production of individual recombinant mucosal carbohydrases that should provide an easier handling, as well as a more predictable and controllable enzyme activity (23). Likewise, estimations of the relative amount of the different mammalian mucosal carbohydrases have been already reported (35). These

values could be useful to establish a physiologically relevant ratio among the different mucosal carbohydrases. Thus, the sucrase-isomaltase complex seems to be the major mucosal carbohydrase, followed by the maltase-glucoamylase complex, whereas the trehalase is the less abundant enzyme (36). Finally, a key aspect that could help to drive harmonization and allow more effective comparison of different *in vitro* digestibility conditions would be to include appropriate suits of control digestible carbohydrates (for instance, maltose, sucrose, starch-derived oligosaccharides, lactose, etc.) and well-recognized non-digestible comparator carbohydrates (such as lactulose or fructooligosaccharides).

Although there is still a considerable way to go in the full understanding of the physical and chemical dynamics of mammalian intestinal mucosal carbohydrases, the growing relevance, based on their beneficial impact of human health, of non-digestible carbohydrates emphasizes the necessity of developing a cost-efficient, fit-for-purpose, easily implemented, and physiologically relevant *in vitro* methodology which considers all human carbohydrases involved in dietary carbohydrate digestion.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work has been financed by the Spanish Ministry of Economy, Industry and Competitiveness (Project AGL2017-84614-C2-1-R).

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Conflict of Interest Statement: The 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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 05 November 2018
doi: 10.3389/fmicb.2018.02558

Mechanistic Insights Into the Cross-Feeding of *Ruminococcus gnavus* and *Ruminococcus bromii* on Host and Dietary Carbohydrates

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Dietary and host glycans shape the composition of the human gut microbiota with keystone carbohydrate-degrading species playing a critical role in maintaining the structure and function of gut microbial communities. Here, we focused on two major human gut symbionts, the mucin-degrader *Ruminococcus gnavus* ATCC 29149, and *R. bromii* L2-63, a keystone species for the degradation of resistant starch (RS) in human colon. Using anaerobic individual and co-cultures of *R. bromii* and *R. gnavus* grown on mucin or starch as sole carbon source, we showed that starch degradation by *R. bromii* supported the growth of *R. gnavus* whereas *R. bromii* did not benefit from mucin degradation by *R. gnavus*. Further we analyzed the growth (quantitative PCR), metabolite production (^{1}H NMR analysis), and bacterial transcriptional response (RNA-Seq) of *R. bromii* cultured with RS or soluble starch (SS) in the presence or absence of *R. gnavus*. In co-culture fermentations on starch, ^{1}H NMR analysis showed that *R. gnavus* benefits from transient glucose and malto-oligosaccharides released by *R. bromii* upon starch degradation, producing acetate, formate, and lactate as main fermentation end-products. Differential expression analysis (DESeq 2) on starch (SS and RS) showed that the presence of *R. bromii* induced changes in *R. gnavus* transcriptional response of genes encoding several maltose transporters and enzymes involved in its metabolism such as maltose phosphorylase, in line with the ability of *R. gnavus* to utilize *R. bromii* starch degradation products. In the RS co-culture, *R. bromii* showed a significant increase in the induction of tryptophan (Trp) biosynthesis genes and a decrease of vitamin B12 (VitB12)-dependent methionine biosynthesis as compared to the mono-culture, suggesting that Trp and VitB12 availability become limited in the presence of *R. gnavus*. Together this study showed a direct competition between *R. bromii* and *R. gnavus* on RS, suggesting that *in vivo*, the *R. gnavus* population inhabiting the mucus niche may be modulated by the supply of non-digestible carbohydrates reaching the colon such as RS.

Keywords: cross-feeding, gut bacteria, *Ruminococcus*, mucin, resistant starch

Abbreviations: Padj, adjusted *p*-value; RS, resistant starch; SS, soluble starch.

OPEN ACCESS

Edited by:
Robert Rastall, University of Reading, United Kingdom

Reviewed by:
Glenn Gibson, University of Reading, United Kingdom
Frank O'Neill Aylward, Virginia Tech, United States

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Specialty section:
This article was submitted to Systems Microbiology, a section of the journal Frontiers in Microbiology

Received: *20 July 2018*
Accepted: *08 October 2018*
Published: *05 November 2018*

Citation:
Crost EH, Le Gall G, Laverde-Gomez JA, Mukhopadhya I, Flint HJ and Juge N (2018) Mechanistic Insights Into the Cross-Feeding of Ruminococcus gnavus and Ruminococcus bromii on Host and Dietary Carbohydrates. Front. Microbiol. 9:2558. doi: 10.3389/fmicb.2018.02558

INTRODUCTION

The human gut is heavily populated by a diverse microbial community (gut microbiota) which plays a crucial role in maintaining human health through, e.g., polysaccharide digestion, metabolite and vitamin production, maturation of the immune system and protection against pathogens (for a review, see Thursby and Juge, 2017). The adult gut microbiota is dominated by members of Firmicutes and Bacteroidetes phyla although organisms from the Actinobacteria, Verrucomicrobia and Proteobacteria phyla also contribute to the structure and function of this microbial community. The microbiota composition varies longitudinally along the gastrointestinal tract but also transversally from the lumen to the mucosa (Donaldson et al., 2016). The colon is the most densely colonized part of the gut, reaching density of 10^{11}–10^{12} cells per gram. The lumen of the gut is generally considered to host a microbial community which is distinct from that of the mucus layer although partial mixing and dispersal by host factors tend to homogenize the community (Mark Welch et al., 2017; Tropini et al., 2017). Several factors influence the biogeography of symbiotic bacteria within the gut, including the gradient and availability of glycans within discrete physical niches (Koropatkin et al., 2012).

In the colon, bacteria have access to non-digestible polysaccharides from the diet but also to complex oligosaccharides from the host mucins (Koropatkin et al., 2012; Tailford et al., 2015a). It is commonly accepted that diet is the main contributing factor influencing the structure of the gut microbial community in the colon (for a review, see Flint et al., 2017). Dietary alteration in the gut microbiota profile can be temporal (e.g., David et al., 2014) or long-term (e.g., Sonnenburg et al., 2016). One of the largest single source of energy for microbial growth in the human colon is dietary starch that escapes digestion in the upper gut and reaches the colon undigested. The fermentation of these substrates provides nutrients for the gut bacteria and short-chain fatty acids (SCFAs). SCFAs are beneficial for colon health; they are a source of energy for the colonocytes and contribute to the maintenance of gut barrier function, the protection against colorectal cancer development and the control of intestinal inflammation (Flint et al., 2017).

Ruminococcaceae are an important family of Firmicutes bacteria within the colonic microbial communities which have evolved specialized systems to utilize complex carbohydrates. This is in contrast to *Bacteroides* which have been shown to display diverse and versatile glycan metabolizing capabilities (for a recent review, see Ndeh and Gilbert, 2018). Members of the genus *Ruminococcus* have been reclassified into three genera and families based on 16S rRNA sequencing, *Blautia* (Lachnospiraceae), *Ruminococcus* (Ruminococcaceae) and *Clostridium* (Clostridiaceae) (Liu et al., 2008). *R. bromii* is one of the most abundant bacteria constituting the human colonic microbiota and a primary degrader of RS, an important non-digestible dietary polysaccharide (Ze et al., 2012, 2013). *Ruminoccocus gnavus* was first assigned as a novel species in 1976 (Moore et al., 1976) and recently reclassified into genus *Blautia* which belongs to *Clostridium* cluster XIVa, a member of the Lachnospiraceae family but still maintaining its original name (Lawson and Finegold, 2015). *R. bromii* and *R. gnavus* are prevalent species of the human gut; They are among the 57 species detected in more than 90% of human fecal samples by metagenomic sequencing (Qin et al., 2010). The median abundances of *R. bromii* L2-63 and *R. gnavus* are around 3 and 0.1%, respectively (Qin et al., 2010). In our previous work we showed that *R. gnavus* ability to grow on host mucin glycoproteins was strain dependent (Crost et al., 2013, 2016), underscoring the importance of analyzing glycan utilization by members of the human gut microbiota at the strain level. The mucin-degrading strain *R. gnavus* ATCC 29149 utilizes mucin glycan epitopes from the intestinal mucus layer as energy source (Crost et al., 2013, 2016).

It has been proposed that the primary role played by *R. bromii* is to release energy from RS to other members of the microbial community (Ze et al., 2012). Trophic interactions between members of the microbiota encompass both cooperation and competition. For example, mucin cross-feeding has been reported between gut microbiota species such as infant bifidobacteria and *Eubacterium hallii* (Bunesova et al., 2018) or *Akkermansia muciniphila* and non-mucus-degrading bacteria *Anaerostipes caccae*, *Eubacterium hallii*, or *Faecalibacterium prausnitzii* (Belzer et al., 2017; Chia et al., 2018). Examples of cross-feeding have also been reported within the *Bifidobacterium* genus (Milani et al., 2015; Turroni et al., 2017), and in the presence of primary degraders of RS (with *R. bromii*) or xylan (with *B. ovatus*) (Turroni et al., 2010, 2012; Rogowski et al., 2015; Centanni et al., 2017).

Here, we investigated the molecular mechanisms underpinning the trophic interactions between the human gut symbionts *R. bromii* and *R. gnavus* on host mucin and dietary starch using a combination of bioinformatics, quantitative PCR (qPCR), NMR-based metabolite profiling and RNA-Seq based transcriptomics of mono- and co-cultures.

MATERIALS AND METHODS

Materials

D-glucose (Glc), type III pig gastric mucin (PGM), maltose, maltotriose and soluble potato starch (SS) were purchased from Sigma-Aldrich (St Louis, MO, United States). Purified pig gastric mucin (pPGM) was prepared as previously described (Gunning et al., 2013). Maltotetraose was obtained from Carbosynth (Berks, United Kingdom). A retrograded type-III RS derived from high amylose maize was kindly provided by Ingredion (Manchester, United Kingdom).

Bacterial Strains and Growth Conditions

Ruminococcus gnavus ATCC 29149 was routinely grown in an anaerobic cabinet (Don Whitley, Shipley, United Kingdom) in Brain Heart Infusion broth supplemented with yeast extract and hemin (BHI-YH) as previously described (Crost et al., 2013). *R. bromii* L2-63 was also grown in an anaerobic

cabinet, in anaerobic basal Yeast extract-Casitone-Fatty Acids (YCFA) medium (Duncan et al., 2002) supplemented with 0.5% SS. Growth of both bacteria on single carbon sources utilized YCFA medium supplemented with 0.5% (wt/vol) of Glc or starch (SS or RS), malto-oligosaccharides at a concentration of 27.7 mM Glc units, or 1% (wt/vol) pPGM. The growth assays were performed in 96-well plates with 200 μL of medium/well for screening or in 14 mL-tubes with 10 mL medium/tube for sampling. Growth was determined spectrophotometrically by monitoring changes in optical density (OD) at 595 nm or 600 nm compared to the same medium without bacterium ($\Delta OD_{600\ nm}$) hourly for the first 10 h and then at distinct times up to 75 h. Sampling for DNA extraction, RNA extraction or ^{1}H NMR was carried out over growth.

DNA Extraction and qPCR

For the isolation of *R. gnavus* ATCC 29149 and/or *R. bromii* L2-63 chromosomal DNA, cells from a 2 mL-aliquot of culture were harvested by centrifugation (10,000 g, 5 min, 4°C), at different times of growth. The cell pellet was kept frozen at −20°C until DNA extraction. The DNA extraction was carried out using Gene JET Genomic DNA Purification kit (ThermoFisher Scientific) following the supplier's procedure for Gram-positive bacteria, except for the elution step which was performed with 100 μL of EB buffer instead of 200 μL. DNA quality and quantity were assessed using the NanoDrop™ 2000 spectrophotometer (ThermoFisher Scientific) and the Qubit dsDNA HS assay on Qubit® 2.0 fluorometer (ThermoFisher Scientific). Dilutions at 10 ng/μL were prepared in water then the DNA was diluted further in 5 μg/ml Salmon Sperm DNA to obtain a 1 ng/μL dilution used as template for qPCR (see below).

The 16S rRNA genes of *R. gnavus* ATCC 2949 and *R. bromii* L2-63 were amplified with universal primers 27F (5″- AGAGTTTGATCMTGGCTCAG- 3″) and RP2 (5″-ACGGCTACCTTGTTACGACTT-3″). The PCR products were purified, quantified and diluted in water to 16.4 ng/μL which equals to 10^{10} copies/μL. A series of 10-fold or 20-fold dilutions was then performed from 10^{10} copies/μL to 10^2 copies/2 μL using 5 μg/mL Salmon Sperm DNA. Calibration curves were prepared in triplicates for each pair of primers using 10^7 copies/2 μL to 10^2 copies/2 μL dilutions of 16S PCR products. The standard curves showed a linear relationship of log input 16S copy number vs. the threshold cycle (C_T), with acceptable values for the slopes and the regression coefficients (R^2). The dissociation curves were also performed to verify the specificity of the amplicons.

Quantitative PCR was carried out in an Applied Biosystems 7500 Real-Time PCR system (Life Technologies Ltd). Three pairs of primers targeting 16S rRNA gene were used in this study (**Supplementary Table S1**). Each qPCR reaction (10 μL) was then carried out in triplicates with 2 μL of DNA matrix at 1ng/μL and 0.2 mM of each primer, using the QuantiFast SYBR Green PCR kit (Qiagen) according to supplier's advice (except for the combined annealing/extension step which was extended to 35 s instead of 30 s).

RNA Extraction, Ribodepletion and RNA-Seq

Total RNA was extracted from 5 mL of mid- to late exponential phase cultures of *R. gnavus* ATCC 29149 and/or *R. bromii* L2-63 in YCFA supplemented with a single carbon source (Glc, SS or RS). Four biological replicates were performed for each carbon source. The RNA was stabilized prior to extraction by adding 1/5 vol of phenol (pH 4.3): ethanol (1:9) mixture to 1 vol of culture then incubating 30 min on ice and finally pelleting the cells for 5 min at 10,000 g at 4°C. Cell pellets were stored at −80°C before extraction. The extraction was performed by a method using phenol and chloroform and adapted from Sambrook et al. (1989). Genomic DNA contamination was removed by DNAse treatment using the TURBO DNA-free kit (Life Technologies Ltd., Paisley, United Kingdom) according to the supplier's recommendations. The purity, quantity and integrity of the DNase-treated RNA were assessed with NanoDrop 2000 Spectrophotometer and with Agilent RNA 600 Nano kit on Agilent 2100 Bioanalyzer (Agilent Technologies, Stockport, United Kingdom). Ribodepletion was then carried out using Ribo-Zero rRNA Removal kit for bacteria according to supplier's advice (Illumina, Cambridge, United Kingdom); efficiency assessment of the ribodepletion was performed by quantifying RNA before and after rRNA removal using the Qubit RNA HS assay on Qubit 2.0 fluorometer.

The rRNA removal was confirmed with a Nano chip run on a Bioanalyzer 2100 (Agilent). Three out of the four replicates were selected for sequencing for each condition. The resulting ribosomal depleted RNA was then fragmented for 8 min at 94°C using the Elute, Fragment, Prime buffer from Illumina TruSeq RNA kit. These conditions produced final libraries of around 370 bp. The samples were then processed following the standard TruSeq RNA protocol. The 15 Illumina libraries were normalized and equimolar pooled to 11 nM using elution buffer (Qiagen) and run over two lanes of the Illumina HiSeq2500 with a 100 bp paired end read metric.

The library pool was then diluted to 2 nM with NaOH and 5 μL transferred into 995 μL HT1 (Illumina) to give a final concentration of 10 pM. A portion (120 μL) of the diluted library pool was then transferred into a 200 μL-strip tube, spiked with 1% PhiX Control v3 and placed on ice before loading onto the Illumina cBot. The flow cell was clustered using a HiSeq PE Cluster Kit v3 (Illumina PE-401-3001) utilizing the Illumina PE_HiSeq_Cluster_Kit_V3_cBot_recipe_V8.0 method on the Illumina cBot. Following the clustering procedure, the flow-cell was loaded onto the Illumina HiSeq2500 instrument following the manufacturer's instructions with a 101 cycle paired reads and a 7-cycle index read. The sequencing chemistry used was HiSeq SBS Kit v3 (Illumina FC-401-3001) with HiSeq Control Software 2.2.68 and RTA 1.18.66.3. Reads in bcl format were demultiplexed based on the 6 bp Illumina index by CASAVA 1.8, allowing for a one base-pair mismatch per library, and converted to FASTQ format by bcl2fastq. The RNA-Seq reads were aligned against the combined reference of *Ruminococcus_bromii*_l2_63.ASM20987v1.31.dna.genome.fa and *Ruminococcus_gnavus*_atcc_29149.ASM16947v1.31.dna.genome.fa using tophat v2.1.0 with the –max-multihits 1 option.

Read counts were obtained using htseq-count v0.6.1[1]. The differential expression analysis was carried out using the DESeq2 (v1.14.0) package (Love et al., 2014). The transcript counts were used as input for DESeq2 and filtered to remove any transcripts with a total count of 0 or 1 over all the samples. Raw counts were normalized to the effective library size separately for *R. bromii* and *R. gnavus* before carrying out the differential expression analysis using the DESeq function. An padj cut-off of 0.05 was used to determine differential expressed transcripts.

^{1}H Nuclear Magnetic Resonance Analysis (^{1}H NMR)

^{1}H NMR analysis was used to identify the presence, absence, and concentration of several metabolites in the bacterial growth medium of mono- and co-cultures. The spent media were thawed at room temperature and prepared for ^{1}H NMR spectroscopy by mixing 400 µL of spent medium with 200 µL of phosphate buffer (0.26 g NaH_2PO_4 and 1.41 g K_2HPO_4) made up in 100% D_2O (100 mL), containing 0.1% NaN_3 (100 mg), and 1 mM sodium 3-(Trimethylsilyl)-propionate-*d*4, (TSP; 17 mg) as a chemical shift reference. The samples were mixed, and 500 µL was transferred into a 5-mm NMR tube for spectral acquisition. The ^{1}H NMR spectra were recorded at 600 MHz on a Bruker Avance spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) running Topspin 2.0 software and fitted with a cryoprobe and a 60-slot autosampler. Each ^{1}H NMR spectrum was acquired with 512 scans, a spectral width of 12300 Hz and an acquisition time of 2.7 s and delay time of 3 s. The "noesygppr1d" presaturation sequence was used to suppress the residual water signal with a low-power selective irradiation at the water frequency during the recycle delay. Spectra were transformed with a 0.3-Hz line broadening, manually phased, baseline corrected, and referenced by setting the TSP methyl signal to 0 ppm. Metabolites were identified using information found in the Human Metabolome Database[2] and by use of the 2D-NMR methods, COZY, HSQC, and HMBC. The metabolites were quantified using the software Chenomx NMR suite 7.6^{TM}.

Bioinformatics Analysis

Genome mining was performed manually by BLAST using the "align two or more sequences" tool (Boratyn et al., 2013). For each target protein, the query sequence used was the reference protein

[1]http://www-huber.embl.de/HTSeq/doc/count.html

[2]http://www.hmdb.ca/

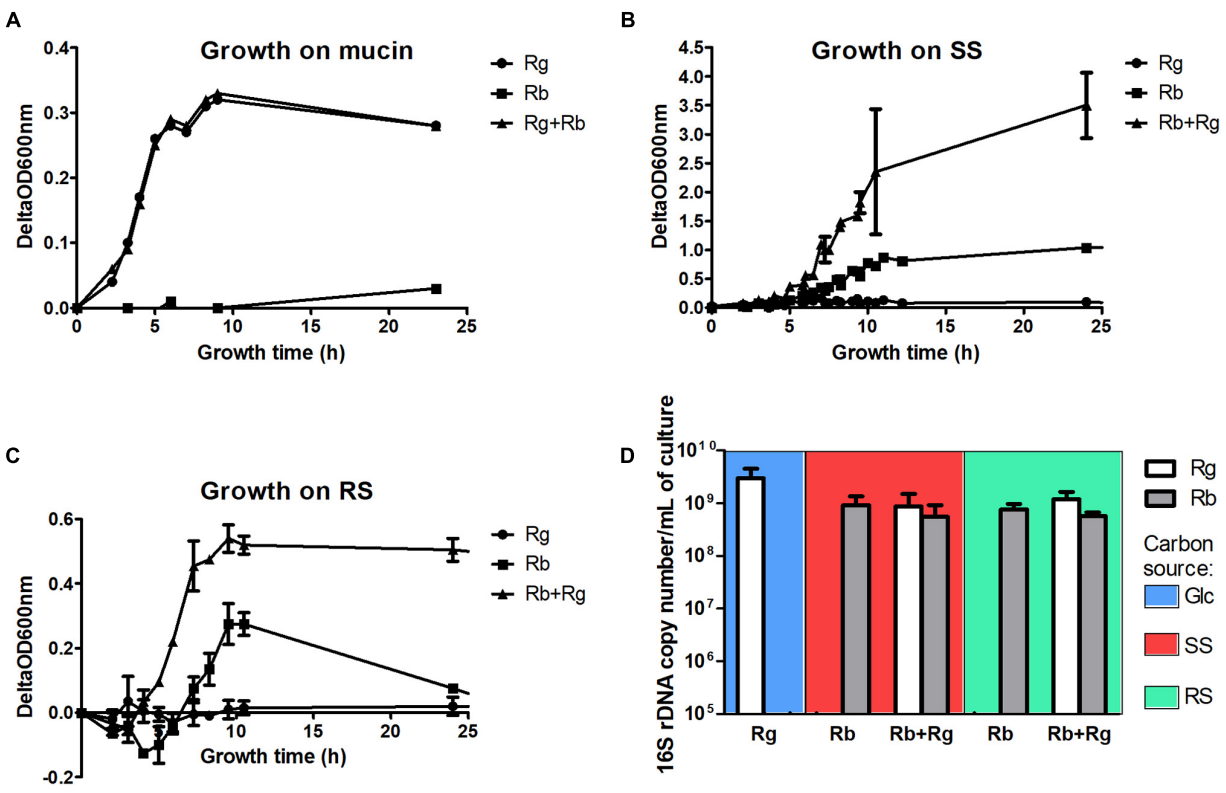

FIGURE 1 | Growth curves of the mono- and co-cultures with mucin **(A)**, soluble starch (SS) **(B)** or resistant starch (RS) **(C)** as sole carbon source and cell concentrations in the different growth conditions **(D)**. The concentrations were determined by qPCR and expressed as 16S rDNA copy number/mL of culture. The values are averages of 3 replicates for *R. gnavus* ATCC 29149 grown with Glc or 4 replicates for the other conditions. The error bars correspond to the standard deviations. Cells samples were collected at a time of growth of 7 h for *R. gnavus* ATCC 29149 grown on Glc, 10 h for *R. bromii* L2-63 grown on SS and 8 h for the other conditions.

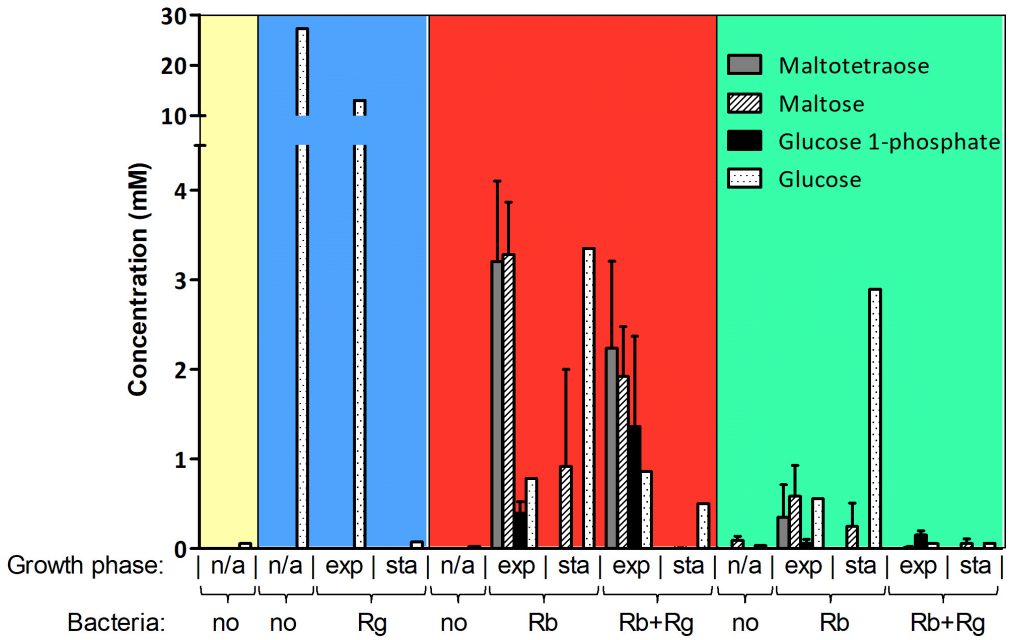

FIGURE 2 | Concentration of starch degradation products in the spent media. The concentrations were determined by ^{1}H NMR and the values are averages of 2 to 5 replicates. The error bars correspond to standard deviations. Results presented in the blue, red and green boxes come from growth assays with Glc, SS and RS as sole carbon source, respectively. Results from the YCFA medium alone, without carbon source, are presented inside the yellow box.
Abbreviations: exp, exponential; sta, stationary; n/a, non-applicable.

sequence from NCBI; when no reference protein sequence was available, a sequence from a member of the Clostridiales order was used (**Supplementary Table S2**). The subject sequences were the sequences corresponding to all putative proteins from *R. bromii* L2-63 or *R. gnavus* ATCC 29149 genomes. The search for NanA, E and K in *R. bromii* L2-63 was performed according to Almagro-Moreno and Boyd (Almagro-Moreno and Boyd, 2009).

RESULTS AND DISCUSSION

R. gnavus Utilizes Starch Degradation Products Released by *R. bromii*

The trophic interactions between *R. bromii* L2-63 and *R. gnavus* ATCC 29149 on host and dietary carbon sources were determined under anaerobic conditions using YCFA as a suitable minimum medium for both strains (Duncan et al., 2002). The growth of *R. bromii* L2-63 and *R. gnavus* ATCC 29149 in mono- or co-cultures was first monitored spectrophotometrically using mucin as sole carbon source. While *R. gnavus* could utilize this substrate, as previously reported (Crost et al., 2013), *R. bromii* was unable to grow on mucin as sole carbon source and no growth benefit was observed on this substrate in the presence of *R. gnavus* (**Figure 1A**). Mucin degradation by bacteria relies on the expression of glycoside hydrolases (GHs)[2] (Lombard et al., 2014) such as sialidases (GH33), α-fucosidases (GH29, GH95), exo- and endo-β-N-acetylglucosaminidases (GH84 and GH85), β-galactosidases (GH2, GH20, GH42), α-N-acetylglucosaminidases (GH89), endo-β1,4-galactosidases (GH98) or α-N-acetylgalactosaminidases (GH101 and GH129) (Tailford et al., 2015a). We previously showed that the ability of *R. gnavus* to grow on mucin was dependent on the expression of a GH33 intramolecular *trans*-sialidase (Crost et al., 2013, 2016; Tailford et al., 2015b) and that fucose was released from mucin by the action of GH29 and GH95 fucosidases (Crost et al., 2013). In contrast, the *R. bromii* L2-63 genome encodes a small number of GHs (Mukhopadhya et al., 2018) compared to *R. gnavus* ATCC 29149 (Crost et al., 2013) (21 in *R. bromii* L2-63 vs. 60 in *R. gnavus* ATCC 29149) and no genes encoding mucin-degrading enzymes were found, in line with the inability of this strain to grow on mucin. In addition, its lack of growth in co-culture with *R. gnavus*, suggests that *R. bromii* cannot utilize the monosaccharides released by *R. gnavus*, in agreement with genomic data suggesting that *R. bromii* does not harbor genes involved in fucose or sialic acid metabolism (**Supplementary Figures S1**, **S2**).

Ruminococcus bromii L2-63 is highly specialized in starch degradation, dedicating 15 of its 21 GH-encoding genes to putative GH13 amylases (Ze et al., 2015). Some of these GH13 amylases revealed an organization in "amylosome", contributing to *R. bromii* exceptional ability to degrade dietary RS (Ze et al., 2015; Mukhopadhya et al., 2018). Here we showed that *R. bromii* was able to utilize both starch substrates (SS and RS) as sole carbon source, in agreement with previous reports (Ze et al., 2012, 2015) whereas no growth was detected with *R. gnavus* on these substrates despite the presence of 9 GH13-encoding genes in *R. gnavus* ATCC 29149 genome. Furthermore, while *R. bromii* growth on SS reached $\Delta OD_{600\ nm}$ of ∼0.8–1, after

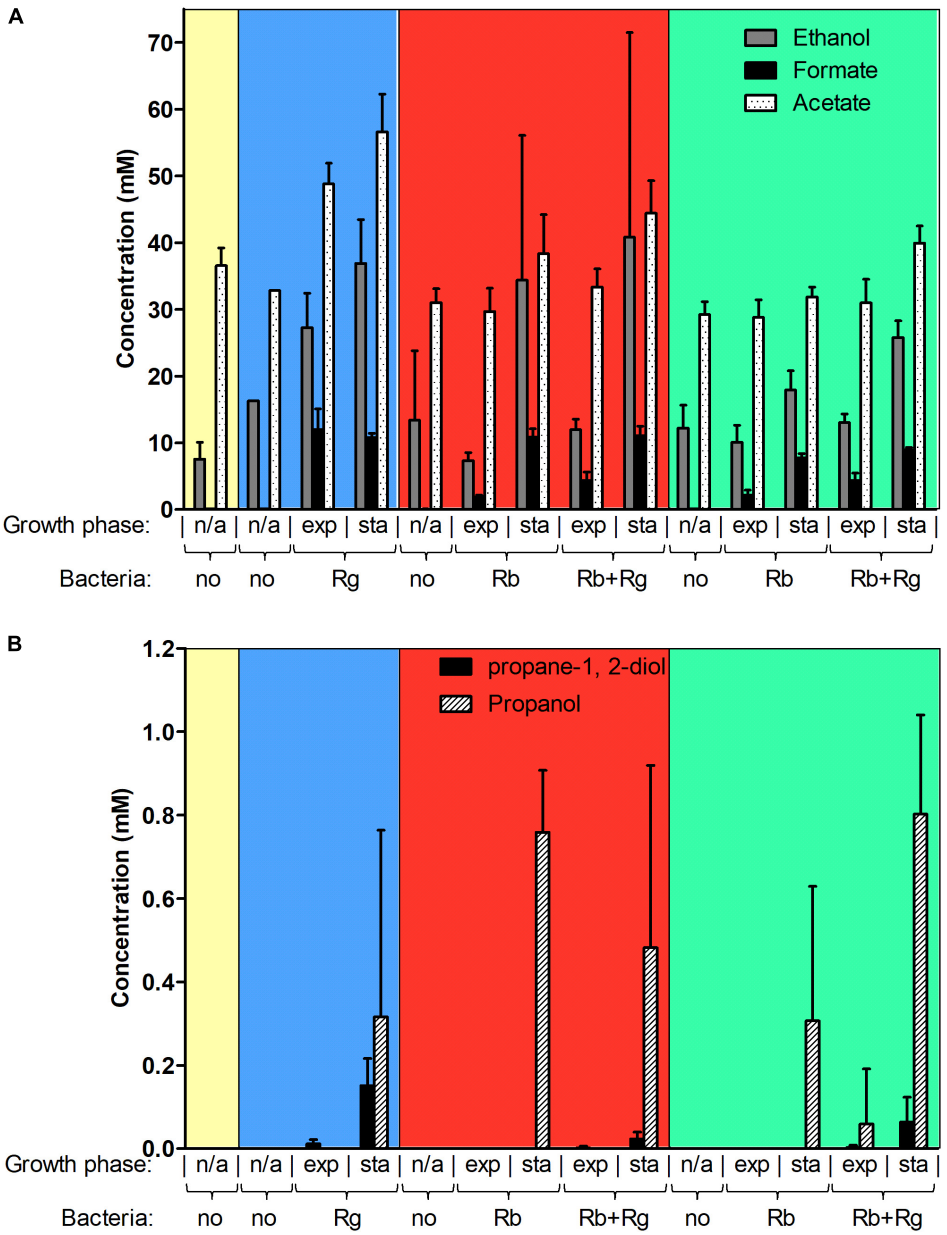

FIGURE 3 | Concentration of different metabolites in the spent media. Concentrations of ethanol, formate and acetate are shown in panel **(A)** while concentrations of propane-1, 2-diol and propanol are shown in panel **(B)**. These concentrations were determined by ^{1}H NMR and the values are averages of 2 to 5 replicates. The error bars correspond to standard deviations. Results presented in the blue, red and green boxes correspond to growth assays with Glc, SS and RS as sole carbon source, respectively. Results from the YCFA medium alone, without carbon source, are presented inside the yellow box. Abbreviations: exp, exponential; sta, stationary; n/a, non-applicable.

12 h of growth, the presence of *R. gnavus* increased the density of cells to $\Delta OD_{600\ nm} \sim 4$ after 10 h, suggesting cross-feeding activity (**Figure 1B**). Due to the presence of insoluble RS particles, the $OD_{600\ nm}$ measurements of *R. bromii* grown on RS result in a two-stage curve reflecting both bacterial growth and bacterial degradation of the RS particles. When *R. gnavus* and *R. bromii* were co-cultured with RS, a different profile was observed (**Figure 1C**), suggesting that cross-feeding also occurs on RS, as confirmed below.

To further assess the behavior of *R. bromii* L2-63 and *R. gnavus* ATCC 29149 on starch (SS or RS), the bacteria were quantified by determining 16S rDNA copies per mL of culture by qPCR (**Figure 1D**). The average 16S rDNA copies of *R. bromii* when grown in mono- or co-cultures with RS after 8 h was 7.3×10^8 and 5.6×10^8 per mL of culture, respectively. When SS was used as the carbon source, *R. bromii* 16S rDNA copies per mL of culture was 1×10^9 after 10 h in mono-culture and 5.8×10^8 after 8 h in co-culture with *R. gnavus*. These analyses indicate

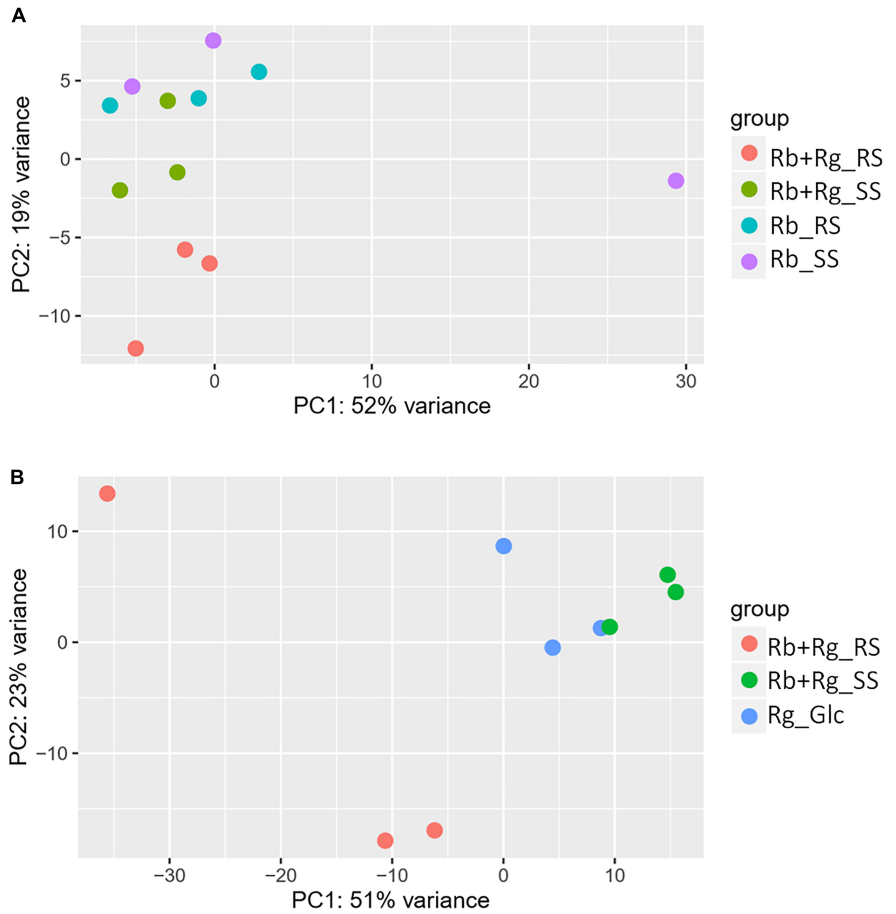

FIGURE 4 | Principal component analysis (PCA) plots for transcriptomics data of *R. bromii* L2-63 genes **(A)** and *R. gnavus* ATCC 29149 genes **(B)**.

that the presence of *R. gnavus* did not affect *R. bromii* growth on starch (RS or SS). *R. gnavus* reached high concentration level in both SS and RS co-cultures with 16S rDNA copies/mL of 6.7×10^8 and 1.2×10^9, respectively, while it was not able to grow in mono-culture on these substrates, confirming that *R. gnavus* benefits from *R. bromii* starch degradation by cross-feeding, as also suggested by spectrophotometric measurements. These concentrations were comparable to the growth of *R. gnavus* on 0.5% Glc as sole carbon reaching 3×10^9 16S rDNA copies/mL after 7 h of growth (**Figure 1D**).

The production and utilization of starch degradation products was monitored over time by ^{1}H NMR (**Figure 2**). Maltotetraose, maltose and glucose-1-phosphate were detected in the spent medium of *R. bromii* mono- or co-cultures (with SS or RS) and their concentration decreased over time. However, while maltotetraose and glucose-1-phosphate were only detected during the exponential phase, maltose was still present at the late stage of growth in mono-cultures. Glc was also released by *R. bromii* degradation of SS or RS but tended to accumulate in mono-cultures while its concentration decreased over time in the presence of *R. gnavus*. Interestingly, the concentration of these starch degradation products was higher in *R. bromii* mono-culture on SS as compared to RS, which may be due to a slower rate of RS degradation allowing a more efficient uptake of the products.

In order to determine which starch degradation products were utilized by *R. bromii* and *R. gnavus*, mono-cultures were performed with malto-oligosaccharides and Glc as control. Both strains could utilize maltose, maltotriose and maltotetraose while Glc was only a substrate for *R. gnavus* (data not shown), suggesting that the release of Glc and malto-oligosaccharides upon *R. bromii* starch degradation contributed to *R. gnavus* cross-feeding on SS or RS. These results suggest that both syntrophy and competition could take place when *R. gnavus* and *R. bromii* are co-cultured with starch.

Effect of Starch Co-cultures on Bacterial Metabolism

Next, we determined the metabolites produced by the bacteria in mono- and co-culture by ^{1}H NMR analysis of the spent media. Acetate was the main SCFA produced by both *R. bromii* L2-63 and *R. gnavus* ATCC 29149 in mono- or co-cultures and its production was increased during bacterial growth (**Figure 3A**). No butyrate or propionate was detected in the

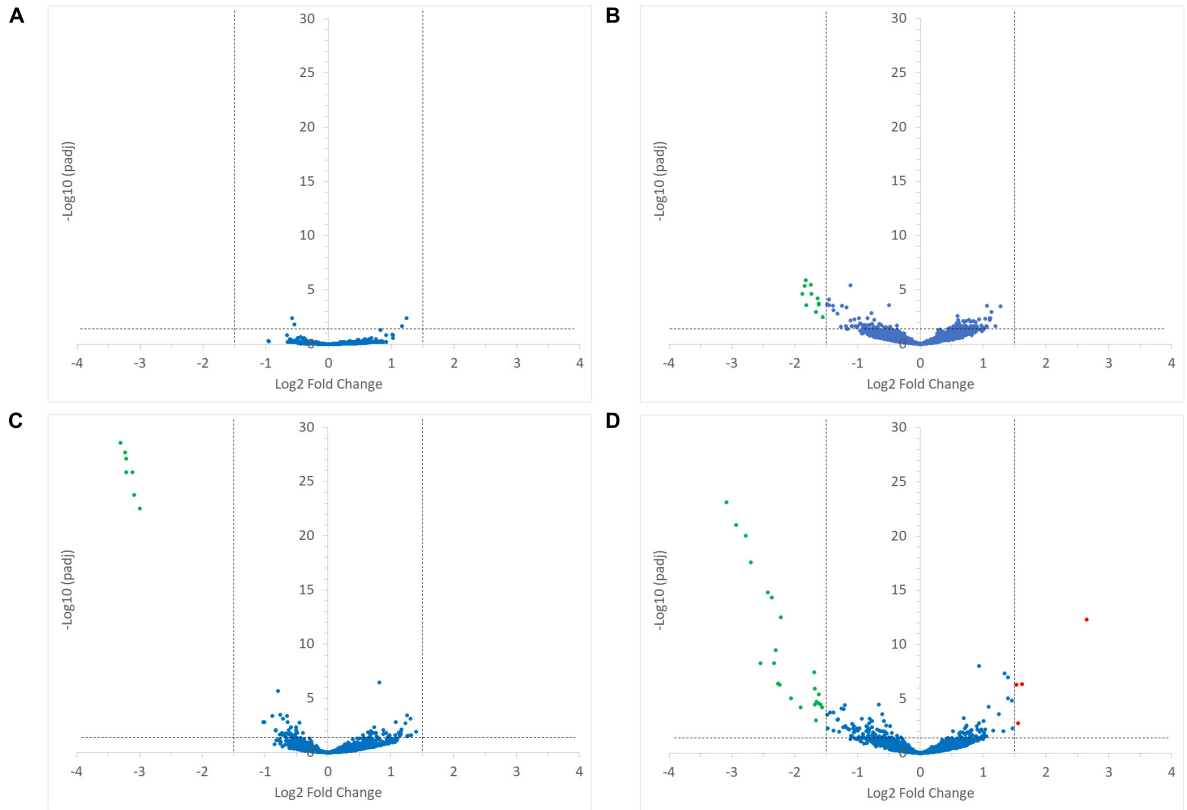

FIGURE 5 | Volcano plots representing the differential expression analysis of *R. bromii* L2-63 genes. Genes are considered to be differentially expressed when Log2 Fold Change < -1.5 or > 1.5 and padj < 0.05; non-differentially expressed genes are shown as blue dots. The impact of starch on *R. bromii* L2-63 gene transcription in mono-cultures and co-cultures is shown in panel **(A,B)**, respectively. **(A)** No gene was differentially expressed between both mono-cultures. **(B)** When comparing the co-cultures, 11 genes were up-regulated with RS as compared to SS (shown as green dots). The impact of *R. gnavus* ATCC 29149 on *R. bromii* L2-63 gene transcription with SS and RS as sole carbon source is shown in panel **(C,D)**, respectively. **(C)** When SS was used as carbon source, 7 genes were up-regulated in the co-culture as compared to the mono-culture (shown as green dots). **(D)** When RS was used as carbon source, 23 genes were up-regulated in the co-culture (shown as green dots) while 4 genes were upregulated in the mono-culture (shown as red dots).

growth conditions tested. Formate and ethanol were produced in increasing amounts by both *R. bromii* L2-63 and *R. gnavus* ATCC 29149 in mono- or co-cultures during bacterial growth (**Figure 3A**). Propanol was detected at low concentration at the late stage of growth when *R. gnavus* was grown with Glc as well as when *R. bromii* was grown with starch in mono- or co-cultures (**Figure 3B**). Interestingly, propanediol was only produced when *R. gnavus* was present (in mono-culture with Glc or in co-cultures with starch), suggesting that propanol is produced via different pathways in *R. bromii* L2-63 and *R. gnavus* ATCC 29149 (**Figure 3B**).

Three main propanol biosynthesis pathways have been identified in bacteria, the propane-1, 2-diol pathway, the acrylate pathway and the Wood-Werkman cycle (Reichardt et al., 2014). These pathways all share the last step, i.e., the conversion of propanal into propanol catalyzed by a propanol dehydrogenase (PduQ). Search for putative enzymes involved in propanol production in *R. gnavus* ATCC 29149 and *R. bromii* L2-63 genomes, identified genes encoding putative PduQ proteins, RUMGNA_01033 and L2-63_01124, respectively. No gene encoding a putative acryloyl-CoA reductase could be found in the genome of these strains, ruling out the acrylate pathway for propanol production in these bacteria. Although both bacteria encode a putative lactaldehyde reductase and a propanol dehydrogenase, PduCDE homologs could only be found in the *R. gnavus* genome indicating that propanol can be produced via the propane-1, 2-diol pathway in this bacterium. This pathway is involved in metabolism of the deoxy-sugars fucose and rhamnose (Reichardt et al., 2014). In addition to PduQ, both bacteria encode homologs of the methylmalonyl-CoA carboxytransferase and propanal dehydrogenase needed for propanol production via the Wood-Werkman cycle (**Supplementary Figure S3**).

Effect of Starch Co-cultures on Bacterial Transcription

To gain further insights into the metabolic pathways underpinning trophic interactions between the two strains, transcriptional analyses of *R. bromii* L2-63 and *R. gnavus* ATCC 29149 grown in mono-cultures on starch (RS or SS) or Glc, respectively, or co-cultures on starch (RS or SS) were performed by RNA-Seq. An average of 20 million reads were generated

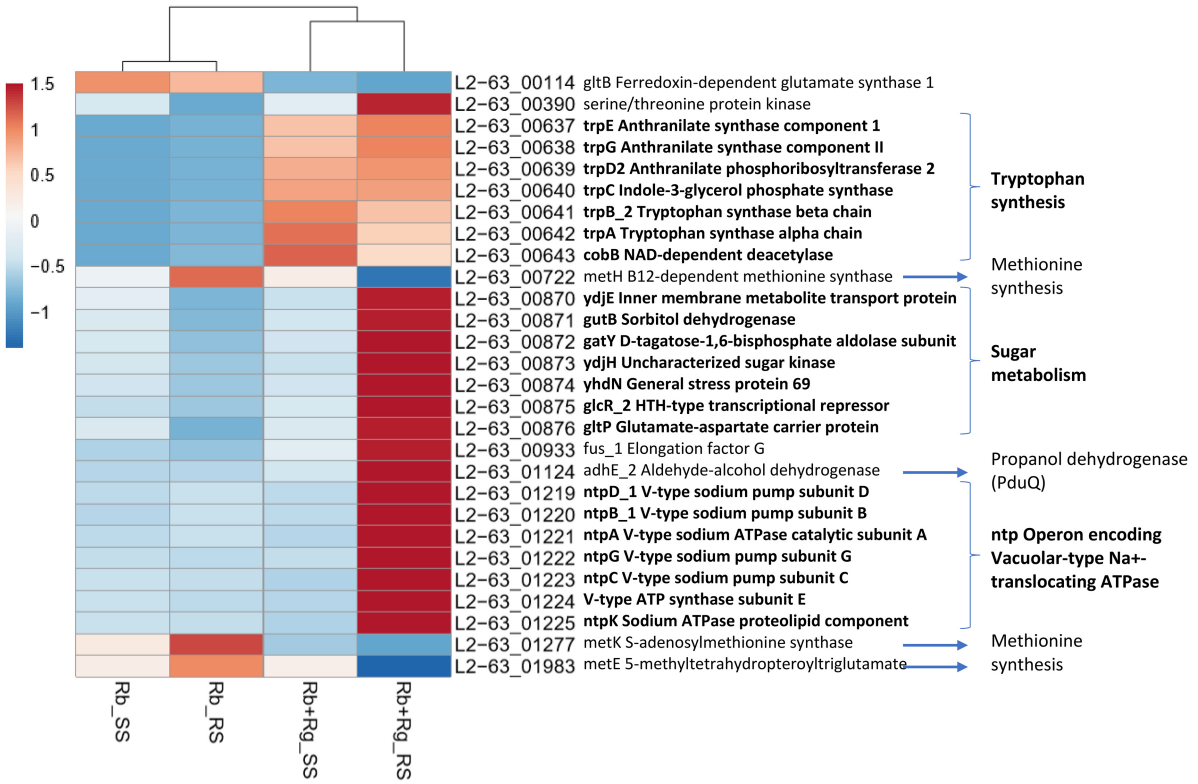

FIGURE 6 | Heatmap of the transcription level (in arbitrary unit) of differentially expressed (Log2 Fold Change < −1.5 or > 1.5 and padj < 0.05) *R. bromii* L2-63 genes in different growth conditions. This heatmap was produced with ClustVis web tool (Metsalu and Vilo, 2015) using the transcript counts as input values.

for each sample which is sufficient sequencing depth. In the co-cultures, the reads assigned to *R. bromii* represented on average around 42% of total reads, for both SS and RS. This result correlates well with the bacterial count determined by qPCR where *R. bromii* 16S copies represented 46% and 32% of total 16S copies in SS and RS, respectively. Differential gene expression analysis (DESeq 2) was carried out to determine the influence of the carbon source or of the other bacterium on gene transcription.

Interestingly, the transcription of all *R. bromii* genes was found to be very similar in both mono-cultures irrespective of the type of starch (**Figures 4A**, **5A**), suggesting that the catabolism of RS or SS shares the same metabolic pathway. For example, the dockerin-carrying amylases Amy4, Amy9, Amy10, and Amy12 GH13 enzymes (Ze et al., 2015) were all expressed in the conditions tested in this study. However, the type of starch had an impact on *R. bromii* gene transcription when in co-culture with *R. gnavus*, with 11 genes up-regulated with RS compared to SS, suggesting a combined effect of RS and *R. gnavus* (**Figure 5B**). These genes belong to 3 different clusters: 3 are part of a cluster of genes potentially involved in sugar metabolism, one is the *pduQ* gene which is involved in the conversion of propanal into propanol (see above) and 7 genes (ntpABCDEGK) are involved in the formation of a ntp sodium pump operon encoding Vacuolar-type Na + -translocating ATPase (**Figure 6** and **Supplementary Table S3**). Interestingly, sodium and potassium ion gradients also serve as important energy reservoirs of bacterial cells and could be upregulated due to the competitive stress in the co-culture (Murata et al., 1996).

In co-cultures, *R. gnavus* had a greater effect than the type of starch on *R. bromii* gene expression. (**Figures 5C,D, 6** and **Supplementary Table S3**). Seven *R. bromii* genes were upregulated in the presence of *R. gnavus* irrespective of the carbon source (**Figure 6** and **Supplementary Table S3**); these genes, which include trpA, B, C, D, E and G, are all involved in the tryptophan biosynthetic pathway and are expressed when tryptophan level is low. In particular trpA, B, C, D, E and G genes were increased by around 13-fold and ninefold in the co-cultures with SS and RS, respectively, as compared to the corresponding mono-cultures. Tryptophan is metabolized by enzymes in the gut mucosa and also by enzymes produced by the gut microbiome. In *R. gnavus* ATCC 29149, RUMGNA_01526 is capable of decarboxylating tryptophan to tryptamine, an activity that is rare among bacteria, and also shared by the common gut Firmicutes member, *Clostridium sporogenes* ATCC 15579 (Williams et al., 2014). It is estimated that ∼10% of the human population harbor one of these enzymes. Interestingly, *R. gnavus* genes involved in tryptophan biosynthesis were not differentially expressed between the three conditions tested, which may be due to *R. gnavus* higher capacity to acquire and metabolize tryptophan from the medium. NMR data confirmed that the tryptophan

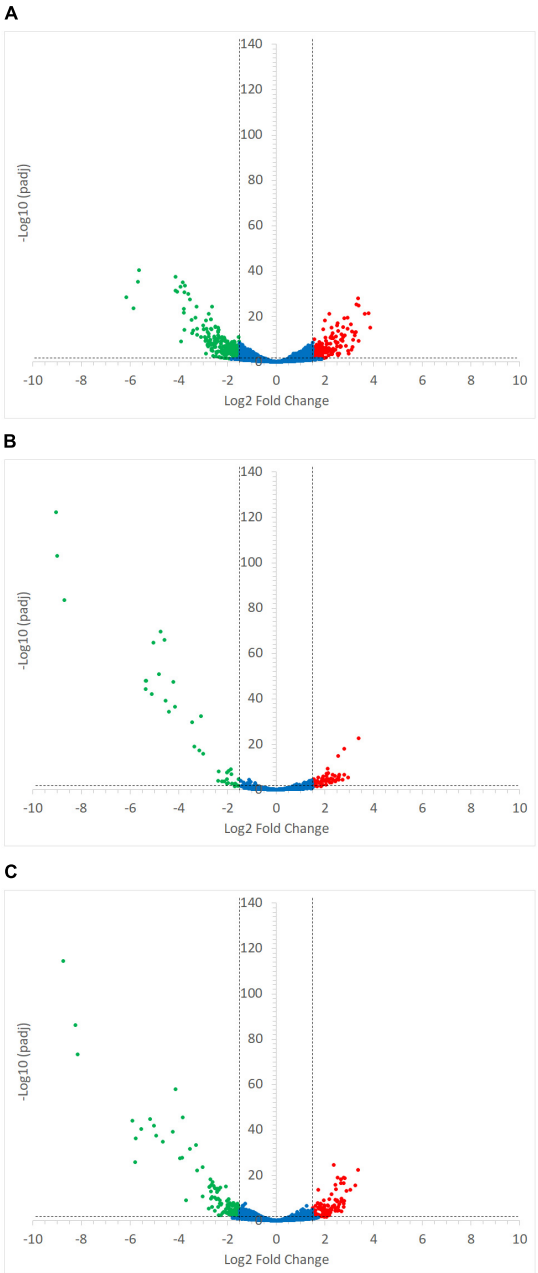

FIGURE 7 | Volcano plots representing the differential expression analysis of *R. gnavus* ATCC 29149 genes. Genes were considered to be differentially expressed when Log2 Fold Change < -1.5 or > 1.5 and padj < 0.05; non-differentially expressed genes are shown as blue dots. Panel **(A)** shows the impact of starch type on *R. gnavus* ATCC 29149 gene transcription when co-cultured with *R. bromii* L2-63; 213 genes were upregulated in the co-culture with RS (shown as green dots) while 212 genes were up-regulated in the co-culture with SS (shown as red dots). The combined effect of the presence of *R. bromii* L2-63 and the carbon source (starch vs. glucose) is shown in panels **(B)** and **(C)** when SS or RS was used in the co-culture, respectively; **(B)** When SS was used as carbon source, 40 genes were up-regulated in the co-culture (shown as green dots) and 59 genes were up-regulated in the mono-culture (shown as red dots). **(C)** When RS was used as carbon source, 119 genes were up-regulated in the co-culture (shown as green dots) while 101 genes were up-regulated in the mono-culture (shown as red dots).

level in the spent medium was lower in the co-cultures as compared to the mono-cultures (data not shown). Together these data suggest that tryptophan may become a limiting factor for *R. bromii* growth on this substrate in the presence of *R. gnavus*. In addition, 16 genes were found to be specifically upregulated in the RS co-culture as compared to the RS mono-culture, including 4 genes belonging to a cluster of genes involved in sugar metabolism (**Figure 6** and **Supplementary Table S3**). It is worth noting that 10 of the 16 genes were also up-regulated in the RS co-culture as compared to SS co-culture. These results further indicate that the observed transcriptional changes in *R. bromii* L2-63 were due to a combined effect of *R. gnavus* and RS.

Interestingly, *R. bromii* showed a down-regulation of the vitamin B12-dependent methionine synthesis genes (metE, metH, metK) in the RS co-culture as compared to the RS mono-culture (**Figure 5D**). The downregulation in co-culture could be due to the lack of sufficient VitB12 (cobalamin) amount in the YCFA growth medium to sustain both *R. bromii* and *R. gnavus* growth as *R. bromii* does not have the ability to produce this vitamin (Ze et al., 2015).

The transcription profile of *R. bromii*, indicate the requirement for *R. bromii* to adjust its metabolic activity toward tryptophan and vitamin B12, especially when RS was used as sole carbon source, so that its growth remains unaffected in the presence of *R. gnavus* as shown above.

Ruminococcus gnavus ATCC 29149 showed a much higher number of differentially expressed genes between mono- and co-cultures as compared to *R. bromii* (**Figure 7**), especially with RS (**Figure 4B**). A total of 22 genes were down-regulated in both co-cultures compared to the mono-culture whereas 20 were upregulated including genes encoding several maltose transporters and enzymes involved in degradation of starch-related products such as RUMGNA_01664 to 01673 and RUMGNA_02728 to 02733 (**Figure 8** and **Supplementary Table S4**). These results are in agreement with the NMR findings showing that *R. gnavus* can utilize malto-oligosaccharides as sole carbon source and from the qPCR analysis showing that *R. gnavus* can efficiently cross-feed on starch-degradation products when grown with *R. bromii*. The fact that, upon *R. bromii* starch degradation, *R. gnavus* benefits from Glc (which is not a substrate for *R. bromii*) may explain why *R. bromii* transcription is not affected on SS (a rapidly degradable starch) in co-culture as this will serve as a preferential nutrient source for *R. gnavus*. However, the ability of *R. gnavus* to utilize malto-oligosaccharides, which is a major nutrient source for *R. bromii* suggests a direct competition between the two strains, which is reflected by *R. bromii* transcriptomics data on RS. This is corroborated by the results of starch degradation products in the spent media (**Figure 2**) which showed Glc presence in SS co-culture medium compared to the absence of Glc in the slow degrading RS co-culture medium.

In summary, we showed that, *in vitro*, *R. gnavus* can efficiently cross-feed on starch degradation products released by *R. bromii*. Cross-feeding plays a crucial role in microbial community shaping in the gut (Hoek and Merks, 2017). This concept involves the ability of bacteria to benefit from substrate degradation

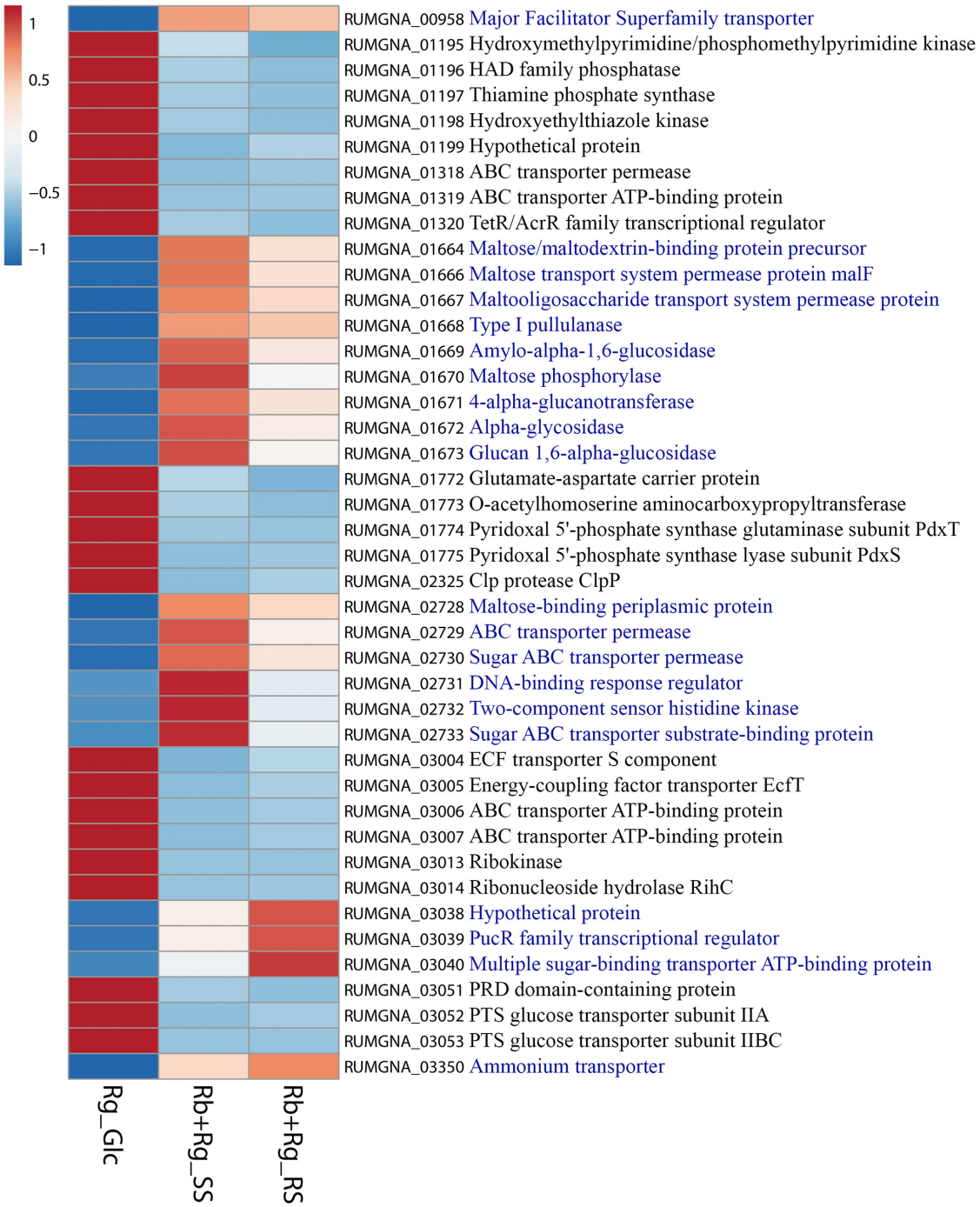

FIGURE 8 | Heatmap of the transcription level (in arbitrary unit) of selected differentially expressed (Log2 Fold Change < −1.5 or > 1.5 and padj < 0.05) *R. gnavus* ATCC 29149 genes in different growth conditions. This heatmap was produced with ClustVis web tool (Metsalu and Vilo, 2015) using the transcript counts as input values. The 20 *R. gnavus* ATCC 29149 genes with an upregulated transcription in both co-cultures with *R. bromii* L2-63 on starch compared to the mono-culture on Glc are in blue. The 22 *R. gnavus* ATCC 29149 genes with an upregulated transcription in the mono-culture on Glc compared to both co-cultures with *R. bromii* L2-63 on starch are in black.

products but also from fermentation products and/or cofactors. For example, *Anaerostipes caccae* L1-92 can utilize both mucin sugars and acetate produced by mucin degradation by *Akkermansia muciniphila* ATCC BAA-835 to sustain its growth and produce butyrate (Chia et al., 2018). Here, we showed that *R. bromii* L2-63 could not benefit from degradation products or metabolites released by *R. gnavus* ATCC 29149 grown on mucin, in line with the unique genomic characteristics of *R. bromii*

strains sequenced to date (Mukhopadhya et al., 2018) and the mucin foraging profile of *R. gnavus* strains (Crost et al., 2013, 2016). However, we showed that RS cross-feeding initiated by *R. bromii* promoted growth of *R. gnavus* leading to the concomitant production of acetate as the main SCFA produced by these strains. Cross-feeding of gut bacteria on starch degradation products has previously been reported *in vitro* between starch degrader *R. bromii* or *Bifidobacterium longum* subsp. *suis* and bacterial species, potentially sharing the same nutrient niche in the gut such as *Anaerostipes hadrus* (Ze et al., 2013) or *B. thermacidophilum* subsp. *porcinum* (Milani et al., 2015), respectively. Resource sharing is an important ecological feature of microbial communities living in the gut (Tannock et al., 2012; Pereira and Berry, 2017; Centanni et al., 2018). The findings from our study suggest that, although *R. gnavus* strains are adapted to the mucosal environment owing to their mucin-foraging capacity (Crost et al., 2013, 2016; Tailford et al., 2015b; Owen et al., 2017), their population dynamics within the colon may also be affected by the supply of dietary carbohydrates that reaches the large intestine undigested such as RS. Due to the high prevalence of *R. bromii* in the human colon, the hydrolysis of RS will cause the release of nutrients such as glucose or metabolites that may reach bacterial species within the mucus layer, potentially promoting the growth of other species to occur, thereby further underscoring the role of *R. bromii* as a keystone species. These findings open the door to future efforts to explore cross-feeding activities between different nutrient niches *in vivo* and the use of RS or other complex polysaccharides as a strategy to address dysbiosis of mucus-associated bacteria associated with human diseases.

DATA AVAILABILITY STATEMENT

The RNA-Seq data generated and analyzed for this study have been deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-7138.

AUTHOR CONTRIBUTIONS

EC carried out most of the practical work (growth assays, DNA extraction, qPCR, RNA extraction, and genome mining) and data analysis. GLG performed the NMR analyses. NJ supervised the research at QIB. IM and HF helped with the analysis of *R. bromii* transcriptomics. EC and NJ wrote the manuscript with contributions from JL-G, IM, and HF.

FUNDING

The authors gratefully acknowledge the support of the Biotechnology and Biological Sciences Research Council (BBSRC), this research was partly funded by the BBSRC Institute Strategic Programs for The Gut Health and Food Safety (BB/J004529/1), Gut Microbes and Health (BB/R012490/1) and Food Innovation and Health (BB/R012512/1) and by the BB/L008602/1 and BB/LL009951/1 BBSRC responsive mode grants.

ACKNOWLEDGMENTS

Next-generation sequencing and library construction was delivered via the BBSRC National Capability in Genomics and Single Cell (BB/CCG1720/1) at the Earlham Institute by members of the Genomics Pipelines Group. We would like to thank Andrea Telatin from the Quadram Institute Bioscience for his help with the RNA-Seq data submission in ArrayExpress.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02558/full#supplementary-material

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Conflict of Interest Statement: The 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.

The reviewer GG and handling Editor declared their shared affiliation.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 04 April 2019
doi: 10.3389/fmicb.2019.00517

In silico Approach for Unveiling the Glycoside Hydrolase Activities in *Faecalibacterium prausnitzii* Through a Systematic and Integrative Large-Scale Analysis

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Systems Microbiology, a section of the journal Frontiers in Microbiology

Received: 06 November 2018
Accepted: 28 February 2019
Published: 04 April 2019

Citation:
Blanco G, Sánchez B, Fdez-Riverola F, Margolles A and Lourenço A (2019) In silico Approach for Unveiling the Glycoside Hydrolase Activities in Faecalibacterium prausnitzii Through a Systematic and Integrative Large-Scale Analysis. Front. Microbiol. 10:517. doi: 10.3389/fmicb.2019.00517

This work presents a novel *in silico* approach to the prediction and characterization of the glycolytic capacities of the beneficial intestinal bacterium *Faecalibacterium prausnitzii*. Available *F. prausnitzii* genomes were explored taking the glycolytic capacities of *F. prausnitzii* SL3/3 and *F. prausnitzii* L2-6 as reference. The comparison of the generated glycolytic profiles offered insights into the particular capabilities of *F. prausnitzii* SL3/3 and *F. prausnitzii* L2-6 as well as the potential of the rest of strains. Glycoside hydrolases were mostly detected in the pathways responsible for the starch and sucrose metabolism and the biosynthesis of secondary metabolites, but this analysis also identified some other potentially interesting, but still uncharacterized activities, such as several hexosyltransferases and some hydrolases. Gene neighborhood maps offered additional understanding of the genes coding for relevant glycoside hydrolases. Although information about the carbohydrate preferences of *F. prausnitzii* is scarce, the *in silico* metabolic predictions were consistent with previous knowledge about the impact of fermentable sugars on the growth promotion and metabolism of *F. prausnitzii*. So, while the predictions still need to be validated using culturing methods, the approach holds the potential to be reproduced and scaled to accommodate the analysis of other strains (or even families and genus) as well as other metabolic activities. This will allow the exploration of novel methodologies to design or obtain targeted probiotics for *F. prausnitzii* and other strains of interest.

Keywords: fermentable sugars, glycoside hydrolases, bioactivity, *Faecalibacterium prausnitzii*, computational screening

INTRODUCTION

The high complexity of the human gut microbiota and the lack of know-how on all the individual players is a key challenge in gut microbiome research. Attempts have been made to define the microorganisms that constitute a healthy human gut microbiota but the scientific community has not yet reached a consensus to define the human beneficial intestinal microbial fingerprint. However, a number of observational studies have repeatedly shown a correlation between some bacterial populations and different physiological states, including those having an influence on human health. Specifically, some groups of strict anaerobes, such as *Faecalibacterium prausnitzii*, are found to be underrepresented in physiological conditions in which inflammation and oxidative stress are present (Joossens et al., 2011; Morgan et al., 2012; Gevers et al., 2014). Thus, during the last decade, *F. prausnitzii* research experienced a boosting, and this microorganism emerged as one of the most promising next-generation probiotics (O'Toole et al., 2017).

The species *Fusobacterium prausnitzii* was reclassified in 2002 by Stewart and co-workers, who proposed the new species *F. prausnitzii* (Stewart et al., 2002). *F. prausnitzii* is highly prevalent in the large intestine of humans and represents a significant proportion of the fecal anaerobes that produce major quantities of butyrate (Sokol et al., 2008; Arumugam et al., 2011). It is part of the autochthonous mucosal bacteria inhabiting the colon and its population has been found to be decreased in several diseases, particularly in those with mucosal inflammation, such as the inflammatory bowel disease or the type 2 diabetes (Ahmed et al., 2007; Sokol et al., 2008; Machiels et al., 2014; Tilg and Moschen, 2014; Rossi et al., 2016). In fact, it was demonstrated that *F. prausnitzii* has anti-inflammatory activity, likely due to its capacity to induce the secretion of the anti-inflammatory cytokine IL-10 in human immune cells and thus modulate T-cell responses (Rossi et al., 2016), as well as to produce microbial anti-inflammatory proteins able to inhibit the NF-κB pathway in intestinal epithelial cells (Quévrain et al., 2016), or extracellular polysaccharides able to attenuate clinical parameters of colitis (Rossi et al., 2015).

Microbiota studies using massive sequencing methodologies, either metataxonomics or metagenomics analyses, point to a high abundance of *Faecalibacterium* in the human gut (Arumugam et al., 2011). However, since *F. prausnitzii* is very sensitive to low oxygen concentrations and possesses a low technological robustness for industrial purposes, it is difficult to isolate from fecal samples and to grow using traditional cultivation methods. Also, although different phylogroups can be distinguished within the species (Benevides et al., 2017), the phenotypic characterization of strains has been restricted to a few isolates.

Metabolic maps have been generated using as the strain A2-165 (DSM17677T) as reference (El-Semman et al., 2014; Rowland et al., 2018), but the *F. prausnitzii* pangenome has never been explored to describe the metabolic capabilities of the genus and be able to develop novel microbial cultivation strategies. Therefore, the aim of the present work was to investigate the genome-based metabolic potential of *F. prausnitzii*, based on a deep prediction of the glycolytic capacities of this bacterium. The *F. prausnitzii* genomes currently deposited in the NCBI Genome Database were explored, taking the metabolisms of *F. prausnitzii* SL3/3 and *F. prausnitzii* L2-6 and data on all known glycoside hydrolases as references. To the best of our knowledge, this is the first time that a high throughput bioinformatics approach is carried out to predict the glycolytic capacities of *F. prausnitzii*.

MATERIALS AND METHODS

The primary aim of the computational workflow developed in this work was to support the investigation of glycoside hydrolase activities in *F. prausnitzii*. Complimentarily, programmatic access to public data sources and the use of broad scope genomic and metabolic tools were sought as means to make this study completely reproducible as well as be able to apply this workflow to similar studies in the future. The proposed workflow (**Figure 1**) encompasses three main steps, i.e., data retrieval, data integration and data analysis. The primary data sources were the carbohydrate-active enzymes database (CAZy), the kyoto encyclopedia of genes and genomes (KEGG), and the Genome database of National center for biotechnology information (NCBI). Data integration was based on the matching of the glycoside hydrolase sequences against the sequences of the proteins encoded in the genomes of *F. prausnitzii* strains. As a rule of thumb, a threshold of 80% was used as baseline to identify most prominent activities in the different strains. Protein homology data were used to segregate clusters of glycoside hydrolases and strains for further pattern analysis (notably, based on the obtained identity percentage). Moreover, gene neighborhood maps were generated for the genes coding for some relevant glycoside hydrolases. Experts curated the results obtained throughout the execution of the workflow towards presenting a comprehensive portrait of current knowledge on glycoside hydrolases activities in *F. prausnitzii* as well as ensuring the validity of the overall process of analysis. The next sections describe the main steps in the workflow, including processing statistics, methods, and **Supplementary Material** produced during the analysis.

Glycoside Hydrolases and Reference Strains

Data on the glycoside hydrolase families were retrieved from the CAZy (Cantarel et al., 2009). From the families present in the CAZy database, only those that have associated fully specified Enzyme Commission numbers (EC number) (Tipton, 1994) were considered (i.e., enzyme codes including four numerical classes separated by periods).

Data on the metabolism of *F. prausnitzii* strains were retrieved from the KEGG (Kanehisa et al., 2017) using its programmatic API. Two strains of *F. prausnitzii* were used as reference in the present study, i.e., *F. prausnitzii* SL3/3 (identified hereinafter as "fpa") and *F. prausnitzii* L2-6 (identified hereinafter as "fpr"). Protein and gene information was retrieved for all the previously selected glycoside hydrolases, including definition (i.e., reference name in GenBank database) (Benson et al., 2018), pathway, amino acid sequence and nucleotide sequence. The KEGG

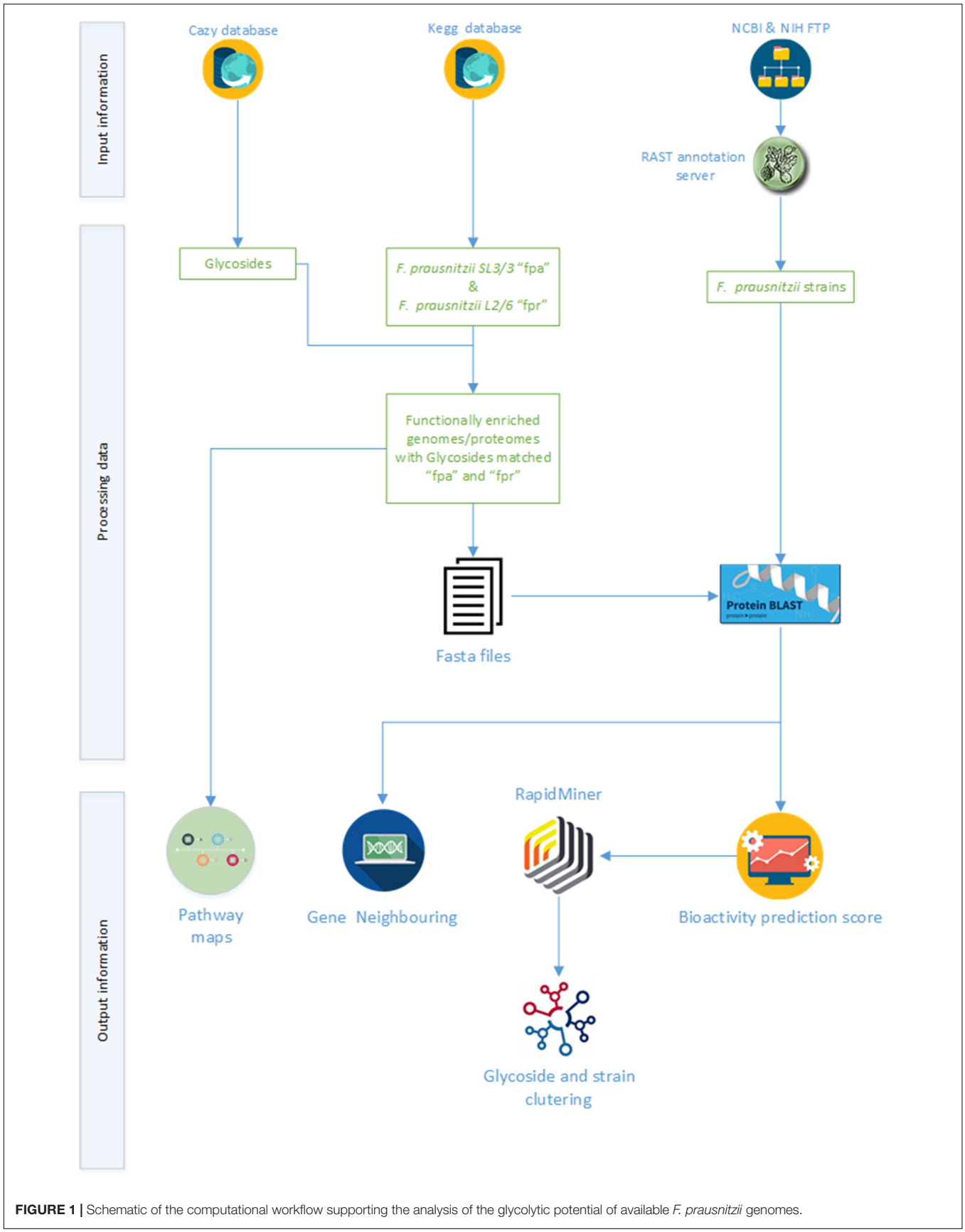

FIGURE 1 | Schematic of the computational workflow supporting the analysis of the glycolytic potential of available *F. prausnitzii* genomes.

Mapper[1] was applied to generate the pathway maps for both strains. The lists of amino acid sequences of glycoside hydrolases found in "fpa" and "fpr" were gathered in two fasta files (see **Supplementary Table S1**).

Publicly available genomes of *F. prausnitzii* strains were retrieved from the Genome database of the NCBI via FTP (Agarwala et al., 2018). From a total of 27 strains, 15 strains have been released in 2017 and the protein annotations are not yet available. Therefore, these genomes required further annotation. The rapid annotation using subsystem technology (RAST) platform was used for this purpose (Overbeek et al., 2014).

Homology Analysis

The basic local alignment search tool (BLAST), most notably the Blastp 2.6.0 tool, supported the search of glycoside hydrolase sequences in the compiled *F. prausnitzii* genomes (Altschul et al., 1997). A Matlab script was coded to generate the results, generally referred to as "fpa" (i.e., *F. prausnitzii* SL3-3 as reference) and "fpr" (i.e., *F. prausnitzii* L2-6 as reference), and obtain the homology data for each of the strains. Specifically, data on the percentage identity, the alignment length, the number of mismatches, gap opens, the start and end of alignment in query/subject, the expected value, and the bit score.

A heatmap-alike representation of the best homology values with associated hierarchical clustering (Euclidean distance and average linkage) was further implemented to support a more intuitive inspection of the results. Values of homology below 50% were suspected to represent non-orthologs enzymes. Conversely, homology values close to 1 were investigated as likely pointing to orthologs enzymes. **Supplementary Table S2** details all these data.

Clustering *F. prausnitzii* Strains

The k-means partitioning clustering method was applied to further analysis of the homology results, most notably to identify groups of strains showing similar traits (Neyman, 1967). This analysis included the clustering of glycoside hydrolases activities according to their prevalence in the analyzed strains as well as the clustering of strains according to the predicted glycoside hydrolases activities.

Distance similarity was based on the Euclidean metric. Several values of k were analyzed for each scenario and the best number of clusters was determined based on the rule of thumb:

$$k \sim \sqrt{n/2} \quad (1)$$

where n being the number of instances to cluster, and the Davies-Bouldin index, which evaluates intra-cluster similarity (i.e., the members of a cluster should present rather similar traits), and inter-cluster differences (i.e., clusters should be reasonably different) (Davies and Bouldin, 1979). Specifically, the number of clusters (k) was initially set to 4 and then fine-tuned using the Davies-Bouldin index (in general, the lowest the better). See **Supplementary Table S3** for details on this analysis.

[1]https://www.genome.jp/kegg/mapper.html

Rapid Miner 8.2 software platform was used to conduct the clustering experiments (Hofmann and Klinkenberg, 2013). The graphical representation of the results was produced using a Python script, based on the graphical libraries NumPy (van der Walt et al., 2011) and Matplotlib (Hunter, 2007).

Gene Neighboring

The SEED genome annotation viewer[2] was used to investigate the gene neighborhoods related to glycoside hydrolase activities. SEED had publicly available annotations for 5 of 27 strains under analysis, i.e., the strains A2-165, M21/2, SL3/3, L2-6, and KLE1255. In-house experts, via the RAST server[3], annotated the other 22 genomes (managed as a private annotation project in SEED). Next, the SEED Viewer tool[4] was used to create a graphical representation of the gene neighborhood for the genes encoding glycoside hydrolases in *F. prausnitzii* or related strains.

Supplementary Table S4 lists the SEED URLs for public inspection. While it is not possible to supply public links for the genomes annotated privately, this document explains how to reproduce the analysis at its full extent.

RESULTS

Metabolic Portrait of *F. prausnitzii*

Currently, KEGG database describes the metabolism of *F. prausnitzii* SL3/3 (identified as "fpa," and documenting 2820 genes and 2756 proteins) and *F. prausnitzii* L2-6 (identified as "fpr," and documenting 2816 genes and 2746 proteins). In particular, KEGG describes 90 metabolic pathways in these organisms. Glycoside hydrolases are annotated in 17 pathways of *F. prausnitzii* SL3/3 and 14 pathways of *F. prausnitzii* L2-6, respectively (**Table 1**). For the most part, glycoside hydrolases are located in the pathways responsible for the starch and sucrose metabolism and the biosynthesis of secondary metabolites.

Figure 2 illustrates the gene orthology and the glycolytic activities in the two strains, as described in KEGG. *F. prausnitzii* SL/3 has 44 glycoside coding genes and *F. prausnitzii* L2-6 has 37 of such genes. 27 of these genes are orthologs, whilst 8 of the genes are specific of *F. prausnitzii* SL/3 and 3 are specific of *F. prausnitzii* L2-6. As a result, the two strains have in common 24 glycoside hydrolases. In terms of specific activity in *F. prausnitzii* SL3/3, this can be pin pointed to the pathways related with sphingolipid metabolism, the pentose and glucuronate interconversions, the glycerolipid metabolism, and the cobalamin biosynthesis (i.e., the glycoside hydrolases 2.4.1.20, 2.4.1.281, 2.4.1.319, 2.4.1.320, 3.2.1.22, 3.2.1.40, and 3.2.1.122). Likewise, the glycoside hydrolases 2.4.1.4, 3.2.1.10, and 3.2.1.26 show activity only in the uridine monophosphate biosynthesis of *F. prausnitzii* L2-6.

[2]http://pubseed.theseed.org/

[3]http://rast.nmpdr.org

[4]http://rast.nmpdr.org/seedviewer.cgi

TABLE 1 | Pathways of the metabolisms of *F. prausnitzii* SL3/3 and *F. prausnitzii* L2-6 containing glycoside hydrolases.

	Number of glycoside hydrolase	
Pathways	**Fpa**	**Fpr**
Starch and sucrose metabolism	17	15
Biosynthesis of secondary metabolites	15	13
Galactose metabolism	7	8
Purine metabolism	6	6
Pyrimidine metabolism	6	6
Sphingolipid metabolism	6	0
Other glycan degradation	4	4
Pentose and glucuronate interconversions	2	2
Cyanoamino acid metabolism	3	2
Pentose and glucuronate interconversions	2	0
Glycerolipid metabolism	2	0
Nicotinate and nicotinamide metabolism	2	2
Porphyrin and chlorophyll metabolism	2	2
Uridine monophosphate biosynthesis, glutamine (+PRPP) ⇒ UMP	0	2
Glycolysis/Gluconeogenesis	3	1
Alanine, aspartate, and glutamate metabolism	1	1
Biosynthesis of antibiotics	1	1
Cobalamin biosynthesis, cobinamide = >cobalamin	1	0

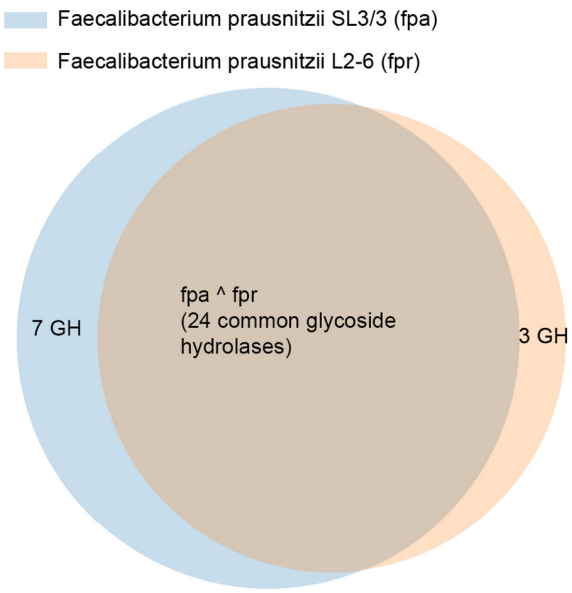

FIGURE 2 | Relation of distribution of glycoside hydrolases in *F. prausnitzii* SL3/3 and *F. prausnitzii* L2-6.

As an example, **Figure 3** details the enzymatic activities found in the starch and sucrose metabolism of *F. prausnitzii* SL3/3. The program implemented in-house to generate the pathway maps using the KEGG Mapper depicts the enzymatic activities of interest as well as organism-specific activity. That is, organism-specific reactions are colored in green and glycoside hydrolases are colored in red. All objects have links to the corresponding KEGG entries.

Homology Analysis

The results of the sequence similarity analysis, in particular the percentage identity data, were further analyzed. **Figure 4** illustrates the results obtained having *F. prausnitzii* SL3/3 (fpa) as reference. **Table 2** describes the glycoside hydrolase activities that have broader, acceptable, and lower probability of being present in the *F. prausnitzii* strains analyzed. A threshold of 50% of homology guided the analysis of gene orthology, i.e., the likelihood of the same gene being present across multiple strains.

Three glycoside hydrolases showed a perfect homology score (98%) for almost all the strains namely: a beta-galactosidase/beta-glucuronidase (FPR_27540) that participates in the galactose and sphingolipid metabolisms (fpa00052 and fpa00600, respectively) as well as in glycan degradation (fpa00511); an uncharacterized peptidyl-tRNA hydrolase (FPR_06900); and, a conserved hypothetical 1,3-beta-galactosyl-N-acetylhexosamine phosphorylase (FPR_27530).

Various other glycoside hydrolases showed homology scores above 75% for (almost) all strains. Notably, a starch synthase (FPR_13470), a cellobiose phosphorylase (FPR_05900), and a 6-phospho-beta-glucosidase (FPR_14930) are highlighted as being potentially present in practically all the studied strains (**Table 2**). The starch and sucrose metabolism (fpa00500) is again in evidence, but these enzymes are also present pathways such as the glycolysis/gluconeogenesis (fpa00010) and the biosynthesis of secondary metabolites (fpa01110).

A predicted 4-O-beta-D-mannosyl-D-glucose phosphorylase (FPR_17220), a 6-phospho-beta-glucosidase (FPR_25170), and a beta-glucosidase (FPR_25480) were among those glycoside hydrolases that showed values of homology below 50%. FPR_17220 is a predicted glycoside hydrolase that was found in only 4 of the strains with a score of no more than 48%. FPR_25170 is a glycoside hydrolase located in the glycolysis/gluconeogenesis metabolism (fpa00010) and the starch and sucrose metabolism (fpa00500), and did not achieve any result above 45%. Finally, FPR_25480 is an aryl-beta-glucosidase, which is present in the starch and sucrose metabolism (fpa00500), the cyanoamino acid metabolism (fpa00460) and the biosynthesis of secondary metabolites (fpa01110). The prediction scores of these glycoside hydrolases were below 45%. Also noteworthy, the aryl-beta-glucosidase (FPR_25480) is the glycoside hydrolase predicted in fewer strains, i.e., 2 out of the 27 strains, even though homology scores are greater than 50%.

The strains CNCM I 4546 and M21/2 showed the most overall homology with respect to the reference strain (SL3/3). They show homology values above 75% for all glycoside hydrolases, with the exception of the 6-phospho-beta-glucosidase (i.e., FPR_25170) in M21/2. Conversely the strain 58_8 showed the lowest overall homology results, i.e., 32 out of the 44 glycoside hydrolases

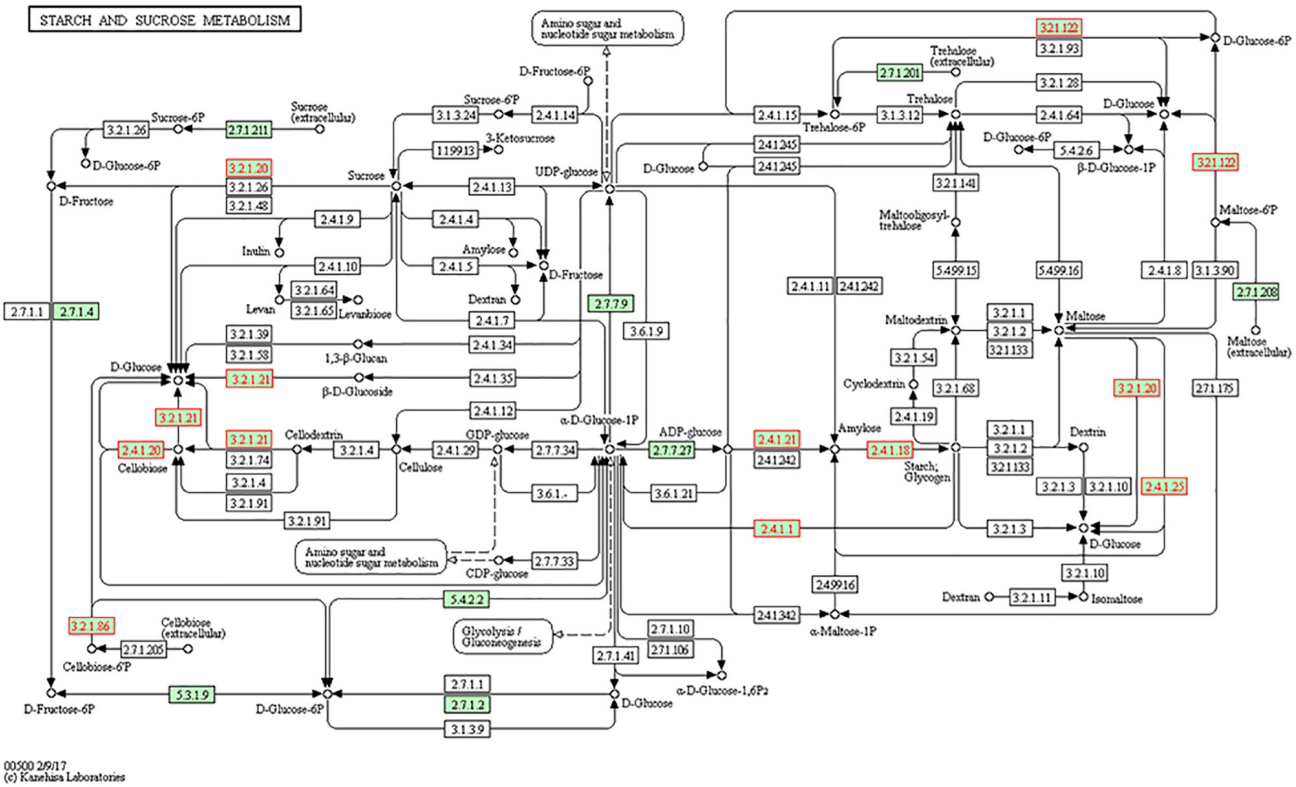

FIGURE 3 | Metabolic map of the starch and sucrose metabolism in *F. prausnitzii* SL3/3. The EC numbers colored in green are organism-specific and red colored font highlights glycoside hydrolase activities

have homology results below 70%. **Supplementary Table S2** details these data.

Clustering of *F. prausnitzii* Strains

Previous works proposed possible divisions of the *Faecalibacterium* group based on phylogenetic analyses (Benevides et al., 2017; Fitzgerald et al., 2018). In this study, the clustering of the studied strains was more specialized, i.e., it was based on the homology results for the glycoside hydrolases. Therefore, here, the strains clustered together have similar homology profiles for the glycoside hydrolases (i.e., glycolytic traits) whereas the clusters represent different, potentially meaningful sets/combinations of glycolytic activities.

Figure 5 describes the clustering of the *F. prausnitzii* strains based on the glycolytic traits as well as phylogeny. The strains present in the glycolytic study are also in the reference phylogenetic studies, with the exception of the strains 2789STDY5834930, 58_8 and 61_16. Color notation helps to compare the present homology-based clustering to two phylogenetic groupings. Overall, most of the groupings are in accordance to one another. The most noticeable difference is that the strain CNCM I 4541 is grouped apart from the rest of the strains showing similar glycolytic traits (i.e., cluster 4, light green colored) in both phylogenetic studies. This glycolytic group showed a high prevalence of glycosides participating in the glycolysis/gluconeogenesis as well as the metabolism of starch and sucrose.

The strains in the cluster 1 (orange colored) and the cluster 2 (pink colored) showed a high frequency of some glycosyltransferases and presence in the pyrimidine metabolism. The biggest difference between these clusters is that cluster 1 shows a low presence of some hexosyltransferases (i.e., FPR_17220 and FPR_17240) whereas cluster 2 is characterized by a low presence of a beta-glucosidase (the FPR_25480), which is present in the starch and sucrose metabolism as well as the cyanoamino acid metabolism.

The strains in the cluster 3 (blue colored) showed low scores for FPR_25170 and high scores for FPR_12120, both glycosides associated with the starch and sucrose metabolism. In turn, the strains of the cluster 4 (light green colored) and the cluster 5 (yellow colored) display overall high frequency profiles. These strains show high homology for enzymes associated to the pyrimidine metabolism. Differences lay in the high score of a 4-alpha-glucanotransferase (FPR_24180) in cluster 4, which participates in the starch and sucrose metabolism, whereas the key glycoside hydrolase in cluster 5 is a purine-nucleoside phosphorylase (FPR_06940), which is part of the metabolisms of purine and the metabolism of nicotinate and nicotinamide.

The clustering model supported by the homology data of *F. prausnitzii* L2-6 (**Figure 6**) is somewhat different from the above described for *F. prausnitzii* SL3-3. The two models present

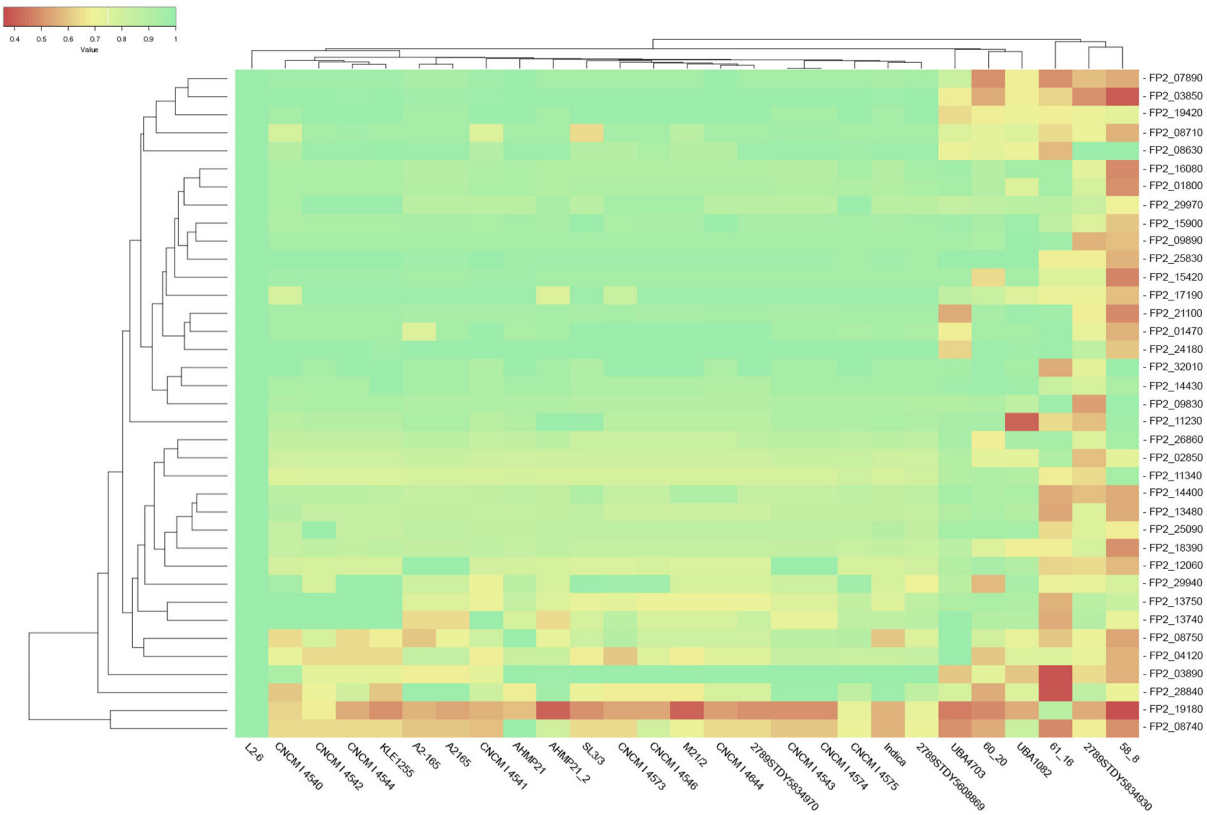

FIGURE 4 | Extract of the heatmap representation of the homology results for *F. prausnitzii* SL3/3. The rows describe glycoside hydrolases and the columns depict the *F. prausnitzii* strains under analysis. The color hue of the cells represents the magnitude of the obtained homology score (red for 0% to green for 100%).

some similar grouping of strains, i.e., the clusters 0 (dark green colored), 3 (blue colored), and 5 (yellow colored). Moerover, the strains *F. prausnitzii* CNCM I 4540, *F. prausnitzii* CNCM I 4542, *F. prausnitzii* CNCM I 4544, *F. prausnitzii* KLEI1255, *F. prausnitzii* UBA4703, and *F. prausnitzii* UBA1082 are grouped together in both models, namely in cluster 2 (pink colored). However, in the first model, this cluster also contains the strain *F. prausnitzii* CNCM I 4541 whereas in the second model this cluster contains the strain *F. prausnitzii* 60_20.

The strains showing the most different/unique traits (i.e., those more distant from the rest of strains) are the same, i.e., the strains *F. prausnitzii* 60_20, *F. prausnitzii* 58_8, *F. prausnitzii* 61_16, and *F. prausnitzii* 2789STDY5834930. Also interesting, the strains *F. prausnitzii* 61_16 and *F. prausnitzii* 60_20 are together when the *F. prausnitzii* SL3-3 is used as reference (cluster 1, colored in orange), whereas, for the *F. prausnitzii* L2-6, the strain *F. prausnitzii* 61_16 is alone (cluster 1, colored in orange), and the strain *F. prausnitzii* 60_20 belongs to cluster 2. Looking into the *F. prausnitzii* SL3-3 homology data, the strains *F. prausnitzii* 61_16 and *F. prausnitzii* 60_20 show similar high homology (>93%) with a predicted unsaturated glucuronyl hydrolase (FPR_02050), an adenine/guanine phosphoribosyltransferase (FPR_06700) and a thymidine phosphorylase (FPR_21760), and similar low homology (<55%) for a 4-O-beta-D-mannosyl-D-glucose phosphorylase (FPR_17220) and a beta-1,4-mannooligosaccharide/beta-1,4-mannosyl-N-acetylglucosamine phosphorylase (FPR_17240). In turn, in the second analysis, the two strains present quite different glycolytic profiles. For example, the homology scores obtained for a glycogen/starch/alpha-glucan phosphorylase (FP2_13480) and an alpha-glucosidase (FP2_19180) differ in 40%.

See **Supplementary Data Sheet S5** for detailed information on this analysis.

Gene Neighboring

Genomic neighborhoods can provide an important level of information about the cooperative role of genes in metabolic routes. Notably, functionally related genes are often organized into co-expression functional networks. Therefore, the study of the gene neighborhoods related to certain glycoside hydrolases, either very frequent or very specific in the present collection of genomes, was interesting to gain a better understanding about the genes that participate in the regulation or are functionally related to those genes encoding these activities. In this regard, the SEED genome annotation viewer is a useful tool to investigate how glycoside hydrolase genes are co-localized with other key genes in the *F. prausnitzii* genomes. As an example, a graphical representation of a potential cellobiose utilization cluster is included in **Figure 7**, showing that genes coding for an ABC transporter, a LacI family transcriptional regulator and

TABLE 2 | Glycoside hydrolases showing very high (>98%), acceptable (>75%), and low values of homology (<50%) having the *F. prausnitzii* SL3/3 strain as reference.

%	Glycoside hydrolase	Number strains	Enzyme	Pathway
>98	FPR_06900	16 strains	EC 3.1.1.29	Non-existent information
	FPR_27540	16 strains	EC 3.2.1.23	Galactose metabolism (fpa00052)
				Other glycan degradation (fpa00511)
				Sphingolipid metabolism (fpa00600)
	FPR_27530	15 strains	EC 2.4.1.211	Non-existent information
>75	FPR_13470	All 27 strains	EC 2.4.1.21	Starch and sucrose metabolism (fpa00500)
				Biosynthesis of secondary metabolites (fpa01110)
	FPR_05900	26 strains (except 60_20)	EC 2.4.1.20	Starch and sucrose metabolism (fpa00500)
	FPR_14930	26 strains	EC 3.2.1.86	Glycolysis/Gluconeogenesis (fpa00010)
		(except 61_16)		Starch and sucrose metabolism (fpa00500)
<50	FPR_17220	L26 58_8 60_20 61_16	EC 2.4.1.281	Non-existent information
	FPR_25170	A2165 CNCM I 4543 CNCM I 4574	EC 3.2.1.86	Glycolysis/Gluconeogenesis (fpa00010) Starch and sucrose metabolism (fpa00500)
	FPR_25480	58_8 60_20	EC 3.2.1.21	Cyanoamino acid metabolism (fpa00460)
				Starch and sucrose metabolism (fpa00500)
				Biosynthesis of secondary metabolites (fpa01110)

a cellobiose phosphorylase (responsible for the phosphorylation of cellobiose and the release of alpha-D-glucose 1-phosphate and D-glucose) seem to be involved in cellobiose uptake. Remarkably, this gene cluster organization is a common feature in other intestinal bacteria (**Figure 7**). **Supplementary Data Sheet S6** presents other examples of gene clusters related to glycoside hydrolase activities in *F. prausnitzii*.

DISCUSSION

Since *F. prausnitzii* emerged as one of the most promising next-generation probiotics, the research efforts invested in gaining a deeper understanding about the metabolism of this microorganism have proliferated. Till date, and to the best of our knowledge, the glycolytic activities of *F. prausnitzii* had not been portrayed.

Therefore, this work presented an *in silico* approach to the systematic and large-scale study of such metabolic activities in the publicly available genomes. Such approach combined sequence similarity analysis, data clustering and gene neighborhood analysis and took advantage on publicly available functional enzyme and pathway annotations. In total, this study screend the activity of 337 glycoside hydrolases in 27 strains, having two possible strains of reference, i.e., the strains *F. prausnitzii* SL3/3 and *F. prausnitzii* L2-6.

Although information about the carbohydrate preferences of *F. prausnitzii* is scarce, the *in silico* metabolic predictions inferred here are consistent with what we know about the impact of fermentable sugars on the growth promotion and metabolism of *F. prausnitzii.* For instance, the broad representation of glycoside hydrolases potentially involved in the metabolism of galactose-containing oligosaccharides points to a theoretical capability to hydrolyse these kind of substrates. Homologs of the beta-D-galactosidase (FPR_27540; EC.3.2.1 23) and the lacto-N-biose phosphorylase (FPR_27530; EC.2.4.1.211) were present in the majority of strains analyzed. Beta-D-galactosidases release terminal non-reducing β-D-galactose residues in galactosides, such as some prebiotic galactooligosaccharides (Garrido et al., 2013), and lactose-N-biose phosphorylase is involved in the metabolism of lacto-N-biose, the major building block of human milk oligosaccharides (Xiao et al., 2010). In this regard, *in vitro* fermentation experiments and *in vivo* intervention studies highlighted the ability of galactooligosacharides to increase the population of *F. prausnitzii* in the human gut microbiota (Azcarate-Peril et al., 2017; Grimaldi et al., 2017). Also, a recent report shows that there is an increase in bacterial taxa belonging to the species *Faecalibacterium* in the gut microbiota of piglets when fed with key human milk oligosaccharides (Jacobi et al., 2016). This suggests that milk oligosaccharides can contribute to the persistence of *F. prausnitzii* in the intestine of suckling pigs, and therefore, trigger the health promoting effects attributed to this bacterium. In relation to the lacto-N-biose phosphorylase activity, it is also worth mentioning that enzymes involved in the galacto N-biose/lacto-N-biose pathway play a crucial role in the metabolism of mucin from epithelial cells, an important colonization factor in intestinal bacteria (Turroni et al., 2010; Duranti et al., 2015).

Other commonly used prebiotic substrates are inulin-type fructans, which are linear fructans with beta (2←1) fructosyl-fructose linkages, including oligofructose (normally with a chain length of 2–8) and inulin (with a chain length up to 60 moieties). A starting alfa-D-glucose moiety can be present in the backbone but it is not essential (Roberfroid, 2007). Inulin-type fructans have been found to be metabolized by *F. prausnitzii* (Dewulf et al., 2013) and to increase the *Faecalibacterium* population within the human microbiota in intervention studies (Ramirez-Farias et al., 2009; Dewulf et al., 2013; Clarke et al., 2017; Healey et al., 2018). In this regard, the proposed bioinformatics approach identified homologs of alfa and beta glucosidases widely distributed in the 27 genomes of the strains analyzed. These enzymes could be involved in inulin-type fructan degradation (Saulnier et al., 2007; Giraldo et al., 2014). Altogether, the broad presence of the glycoside hydrolase activities discussed above in the *F. prausnitzii* genomes suggests that this species possesses ecological traits that favor its adaptation to the gut environment.

Current Analysis		Benevides et al, Frontiers in Microbiology		Fitzgerald et al, BMC Genomics	
Cluster 0	2789STDY5834930	Group A	CNCM I 4644	I	M21/2
	58_8		M21-2		918/95b
Cluster 1	61_16		CNCM I 4546		CNCM I 4546
	60_20		CNCM I 4573		CNCM I 4573
Cluster 2	CNCM I 4542		SL3/3		SL3/3
	KLE1255	Group B	A2-165		924/119
	UBA4703		CNCM I 4543		ATCC 27766
	UBA1082		CNCM I 4574		ATCC 27768T
	CNCM I 4544		HMI 19 (other sequence of strain AHMP21)		2789STDY5834970
	CNCM I 4540	Group C	KLE1255		CNCM I 4644
	CNCM I 4541		CNCM I 4544		923/51-1
Cluster 3	A2-165		CNCM I 4542	IIa	CNCM I 4544
	CNCM I 4543		CNCM I 4540		CNCM I 4542
	CNCM I 4574	No group	CNCM I 4575		KLE1255
	Indica		AHMP-21		CNCM I 4540
	A2165		L2-6		CNCM I 4541
	AHMP21_2		CNCM I 4541	IIb	CNCM I 4574
Cluster 4	CNCM I 4546				CNCM I 4543
	CNCM I 4573				A2-165
	CNCM I 4644				HMI_19
	SL3/3				2789STDY5608869
	M21/2				CNCM I 4575
	2789STDY5834970				922/41-1
Cluster 5	CNCM I 4575				AHMP21
	L2-6				923/61-1
	AHMP21				942/8-14-2
	2789STDY5608869				942/30-2
					L2-6
					942/32-1

FIGURE 5 | Clustering of strains activities having *F. prausnitzii* SL3-3 (fpa) as reference. The reference strains are located in cluster 4. Strain per cluster: cluster 0 (58_8, 2789STDY5834930), in cluster 1 (60_20 and 61_16), in cluster 2 (UBA4703, UBA1080, CNCM | 4544, KLE1255, CNCM | 4542, CNCM | 4540, and CNCM | 4541), in cluster 3 (A2-165, Indica, CNCM | 4574, A2165, CNCM | 4543, and AHMP21-2), in cluster 4 (M21/2, CNCM | 4573, 2789STDY5834970, SL3/3, CNCM | 4546, and CNCM | 4644), and in cluster 5 (CNCM | 4575, L2-6, AHMP21, and 2789STDY5608869).

Current Analysis		Benevides et al, Frontiers in Microbiology		Fitzgerald et al, BMC Genomics	
Cluster 0	2789STDY5834930	Group A	CNCM I 4644	I	M21/2
	58_8		M21-2		918/95b
Cluster 1	61_16		CNCM I 4546		CNCM I 4546
Cluster 2	60_20		CNCM I 4573		CNCM I 4573
	CNCM I 4540		SL3/3		SL3/3
	UBA4703	Group B	A2-165		924/119
	UBA1082		CNCM I 4543		ATCC 27766
	CNCM I 4542		CNCM I 4574		ATCC 27768T
	CNCM I 4544		HMI 19 (other sequence of strain AHMP21)		2789STDY5834970
	KLE1255	Group C	KLE1255		CNCM I 4644
Cluster 3	A2-165		CNCM I 4544		923/51-1
	A2165		CNCM I 4542	IIa	CNCM I 4544
	Indica		CNCM I 4540		CNCM I 4542
	AHMP21_2	No group	CNCM I 4575		KLE1255
	CNCM I 4543		AHMP-21		CNCM I 4540
	CNCM I 4574		L2-6		CNCM I 4541
Cluster 4	SL3/3		CNCM I 4541	IIb	CNCM I 4574
	CNCM I 4573				CNCM I 4543
	CNCM I 4546				A2-165
	2789STDY5834970				HMI_19
	M21/2				2789STDY5608869
	CNCM I 4644				CNCM I 4575
	CNCM I 4541				922/41-1
Cluster 5	AHMP21				AHMP21
	2789STDY5608869				923/61-1
	CNCM I 4575				942/8-14-2
	L2-6				942/30-2
					L2-6
					942/32-1

FIGURE 6 | Clustering of strains having *F. prausnitzii* L2-6 (fpr) as reference. The reference strains is located in cluster 5. Strains per cluster: cluster 0 (58_8 and 2789STDY5834930), in cluster 1 (61_16), in cluster 2 (UBA1082, UBA4703, 60_20, CNCM | 4540, CNCM | 4542, CNCM | 4544, and KLE1255), in cluster 3 (A2165, A2-165, CNCM | 4543, Indica, AHMP-21_2, CNCM | 4574), in cluster 4 (CNCM | 4541, CNCM | 4546, CNCM | 4573, CNCM | 4644, SL3/3, M21/2, and 2789STDY5834970), and in cluster 5 (L2-6, 2789STDY5608869, CNCM | 4575, and AHMP-21).

Moreover, a wide representation of strains within the *F. prausnitzii* species. Furthermore, the gene neighboring analysis enabled the description of the genetic context surrounding glycoside hydrolase genes. In present analysis detected glycoside hydrolases potentially involved in the metabolism of maltose (FPR_07280; EC.3.2.1.20) and cellobiose (FPR_05900; EC.2.4.1.20) in the genomes of all the strains analyzed. Maltose and cellobiose are added in

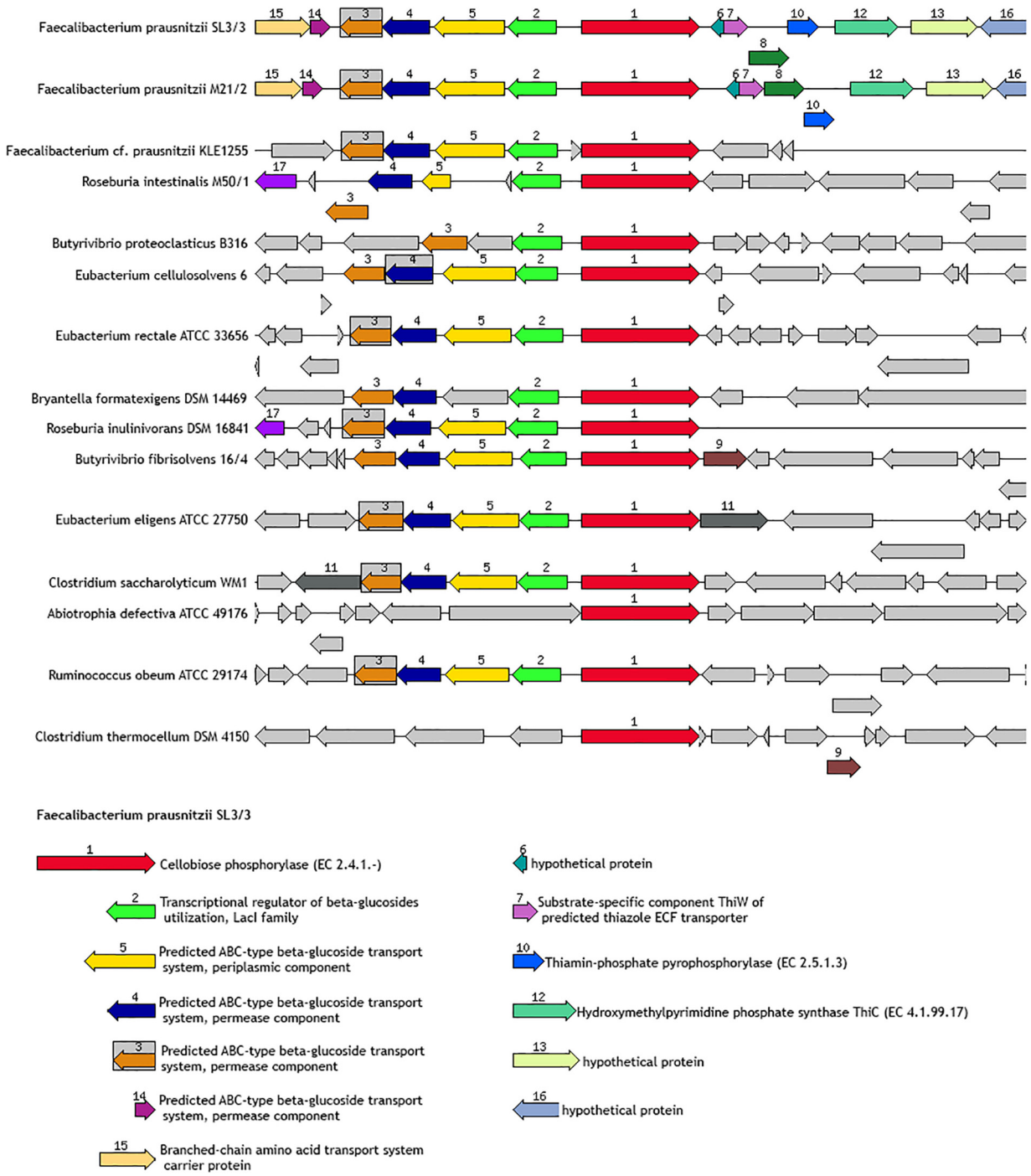

FIGURE 7 | Schematic of the gene neighbor of FPR_05900 (EC.2.4.1.20). This gene encodes a predicted unsatured glucuronyl hydrolase involved in the regulation of bacterial surface properties and related properties.

culture media for *F. prausnitzii* since these disaccharides specifically promote its growth in laboratory conditions (Martín et al., 2017). Our results support the fact that maltose and cellobiose utilization can be a common metabolic characteristic of *F. prausnitzii*, rather than a strain-dependent feature, and highlight the importance of including this kind of carbon sources for the isolation particular, genes potentially involved in oligosaccharide metabolism are organized in clusters, which are specific to the uptake and degradation of the substrates. This is in accordance with what has been described for other intestinal bacteria (Pokusaeva et al., 2011; Wei et al., 2014).

In summary, although the suitability of our *in silico* approach to infer glycoside hydrolase functional maps of *F. prausnizii* needs to be validated using culturing methods, the results showed in this work are in agreement with the current, limited knowledge on carbohydrate metabolism in this species. After experimental validation, our bioinformatics approach may be reproduced and scaled in order to accommodate the analysis of other strains (or even families and genus), as well as other metabolic activities. This will allow the exploration of novel methodologies to design or obtain targeted prebiotics for *F. prausnitzii* and other strains of interest.

AUTHOR CONTRIBUTIONS

BS, FF-R, AM, and AL conceived and designed the study. GB compiled the data and executed all the analyses. All authors drafted the manuscript and read and approved the final version of the manuscript.

FUNDING

This work was supported by the Spanish "Programa Estatal de Investigación, Desarrollo e Innovación Orientada a los Retos de la Sociedad" (Grant AGL2016-78311-R); and, the Asociación Española Contra el Cáncer ("Obtención de péptidos bioactivos contra el Cáncer Colo-Rectal a partir de secuencias genéticas de microbiomas intestinales," Grant PS-2016). This work was also supported by the Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic funding of UID/BIO/04469/2013 unit and COMPETE 2020 (POCI-01-0145-FEDER006684). This work was partially supported by the Consellería de Educación, Universidades e Formación Profesional (Xunta de Galicia) under the scope of the strategic funding of ED431C2018/55-GRC Competitive Reference Group.

ACKNOWLEDGMENTS

SING group thanks CITI (Centro de Investigación, Transferencia e Innovación) from University of Vigo for hosting its IT infrastructure.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00517/full#supplementary-material

TABLE S1 | List of the amino acid sequences of the glycoside hydrolases in two fasta files fpa.fasta (corresponds to *F. prausnitzii* SL3/3) and fpr.fasta (corresponds to *F. prausntizii* L2-6).

TABLE S2 | Extended analysis of the homology among *F. prausntizii* strains.

TABLE S3 | Graphic representation of the clusters and the Davis-Boulding index.

TABLE S4 | List of SEED URLs shown for the gene neighbourhood.

DATA SHEET S1 | Analysis of *F. prausnitzii* gene orthology.

DATA SHEET S2 | Differents examples of gene neighbourhoods related to glycoside hydrolase activities en *F. prausnitzii*.

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Conflict of Interest Statement: BS and AM are on the scientific board and are co-founders of Microviable Therapeutics SL.

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.

frontiers in Nutrition

REVIEW
published: 31 May 2019
doi: 10.3389/fnut.2019.00078

Technological Aspects of the Production of Fructo and Galacto-Oligosaccharides. Enzymatic Synthesis and Hydrolysis

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OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Nutrition and Food Science Technology, a section of the journal Frontiers in Nutrition

Received: *05 February 2019*
Accepted: *15 May 2019*
Published: *31 May 2019*

Citation:
Martins GN, Ureta MM, Tymczyszyn EE, Castilho PC and Gomez-Zavaglia A (2019) Technological Aspects of the Production of Fructo and Galacto-Oligosaccharides. Enzymatic Synthesis and Hydrolysis. Front. Nutr. 6:78. doi: 10.3389/fnut.2019.00078

Fructo- and galacto-oligosaccharides (FOS and GOS) are non-digestible oligosaccharides with prebiotic properties that can be incorporated into a wide number of products. This review details the general outlines for the production of FOS and GOS, both by enzymatic synthesis using disaccharides or other substrates, and by hydrolysis of polysaccharides. Special emphasis is laid on technological aspects, raw materials, properties, and applications.

Keywords: fructo-oligosaccharides, galacto-oligosaccharides, enzymatic synthesis, hydrolysis, properties and applications, alternative substrates

INTRODUCTION

The first reference to prebiotic concept dates from 1954, when Gyorgy reported that a component of human milk (N-acetyl-glucosamine) promoted the growth of a strain from the genus *Bifidobacterium*. A few years later, Petuely (1) recognized lactulose as a *bifidus factor*. Almost 20 years after, Japanese researchers reported that several non-digestible oligosaccharides were *bifidus factors* (2, 3). The term prebiotic as such, was defined in 1995 (4), as "non-digestible food components that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thus improving host health" (5).

Since then, the original definition was subjected to several revisions. According to the most recent one, prebiotics are "substrates that are selectively used by host microorganisms conferring a health benefit" (6). Research in different domains (glycomics, proteomics, etc.), reveals more complex interactions of putative prebiotics with the host, thus this definition is far from being the last one. From a scientific point of view, it is a subject still under development and the advances in this issue impact not only on the scientific community, but also on regulatory agencies, food industries, consumers and healthcare professionals (7).

Regardless the definition, fructo- and galacto-oligosaccharides (FOS and GOS) are widely known because of their prebiotic properties. Additionally, their nutritional properties are also important, they are low caloric sweeteners, give a feeling of satiety, contribute to body weight control, relieve constipation, have a low glycemic index and are not cariogenic (8). GOS and FOS are used in the formulation of dairy products, different types of beverages, bakery products, and some sweets, converting them in functional foods (9). Moreover, they are extensively employed in infant formula to stimulate the development of newborn microbiota (10, 11).

As GOS and FOS can be incorporated in many products, their demand has exponentially increased worldwide over time (12). Japan has been pioneer in the production and consumption of FOS and GOS. It was the first country to incorporate non-digestible oligosaccharides in foods, being a world leader in the use of prebiotics as functional ingredients.

In 2006 the functional food market was estimated to be $20 billion in the United States, $15 billion in Europe, and $12 billion in Japan, growing at an annual rate of 7.5% (13). Particularly the prebiotic market reached $200 million in 2015, with an increase rate of about 15% per year (www.reuters.com/article/pressRelease). What is more, according to Global Market Insights, INC (Delaware, USA), the global prebiotic market is expected to surpass $8.5 billion by 2024 (14). It is remarkable that the increase of the prebiotic market is much higher than that of the food market as a whole, whose increase is about 2% per year.

Considering the economical and nutritional importance of FOS and GOS, this review will be focused on their obtaining. From a technological point of view, these prebiotics can be produced either from natural sources or by enzymatic synthesis using disaccharides or other substrates as raw materials. Furthermore, the hydrolysis of polysaccharides present in many fruits and vegetables is another way for obtaining FOS and GOS. Different methods for producing FOS and GOS will be presented, with special emphasis on raw materials, suitable for both synthesis and hydrolysis reactions. Additional properties and applications of FOS and GOS will be also discussed.

FOS

Fructo-oligosaccharides (FOS) are composed of a small number of fructose units linked by (2→ 1)-β-glycosidic bonds and having a single D-glucosyl unit at the non-reducing end. Particularly, short chain FOS are mixtures of the smallest oligosaccharides, namely 1-kestose [degree of polymerization (DP) equal to 3], nystose (DP4) and 1^F-fructofuranosylnystose (DP5) (4). They can be obtained either by enzymatic synthesis or by hydrolysis of inulin from natural sources mainly from roots of chicory, artichoke, yacon, dahlia or agave. This later method leads to higher molecular weight FOS.

FOS Obtained by Enzymatic Synthesis

The production of FOS obtained by enzymatic synthesis involves transfructosylation reactions where fructosyltransferases (β-fructofuranosidase, EC 3.2.1.26 or β-D-fructosyltransferase, EC 2.4.1.9) act as biocatalysts (10, 11, 15–17). Meiji Seika Kaisha Ltd. pioneered the production of FOS by enzymatic synthesis using the organism of *Aspergillus niger*. Nowadays, this company is one of the leaders of short chain FOS market all over the world, their products are labeled under the brand names Actilight® in Europe and Meioligo in Asia (18). Additionally, NutraFlora® from Ingredion group companies is another brand of short chain FOS that leaders the market in North and South America and Australia (19).

Transfructosylation reactions involve the cleavage of the β-2,1-glycosidic bond and the transfer of fructosyl moieties from carbohydrates acting as donors onto any acceptor other than water (17). Most fructosyltransferases have also a hydrolytic activity, so that the production of FOS is a complex process in which different reactions of synthesis and hydrolysis occur simultaneously both in parallel and in series (17), through consecutive sets of disproportionation reactions. **Figure 1** gives a simplified general outline of the mechanism of the mentioned reactions. In such reactions, the FOS synthesized in the first steps act as fructosyl donors and acceptors leading simultaneously to the production of FOS with DP immediately higher (DPn+1) and lower (DPn-1) than those of the FOS acting as reagents (20). As a result, mixtures of short chain FOS (DP ranging from 2 to 6, i.e., DP3, DP4, DP5, and DP6) (4), together with glucose (secondary product), are obtained. To mathematically describe this mechanism, many authors adapted a kinetic model based on Michaelis-Menten mechanism, assuming that the series of transfructosylation reactions with sucrose, 1-kestose (DP3), and nystose (DP4) as substrates occur in chain, and also considering a competitive glucose inhibition. One of the first approaches in this sense, is the one proposed by Jung et al. (20). They described the reaction mechanism with sucrose as a substrate that can act either as donor or as acceptor, so that 1 mole of glucose and 1 mole of 1-kestose (DP3) are formed simultaneously, indicating a disproportionation reaction mechanism. This pattern was extended to explain the rest of the pathways involved in the course of the synthesis: 1-kestose (DP3) acts as a substrate and sucrose and nystose (DP4) are produced, afterwards nystose (DP4) acts as a substrate and kestose (DP3) and fructofuranosyl nystose (DP5) are formed. Applying mathematical integration of the several reaction patterns proposed, authors were able to calculate the Michaelis-Menten kinetic constant and the maximum rate of appearance of each product. Duan et al. (21), modified this mathematical model by adding the fact that glucose acts as a substrate inhibitor even for sucrose, 1-kestose (DP3) and nystose (DP4). The same kinetic approach was mathematically described by Alvarado-Huallanco and Maugeri Filho (22), using purified and non-purified fructosyltransferase from *Rhodotorula* sp. In this latter model, the authors considered that hydrolysis occurs when nystose (DP4) concentration reaches about 5% (w/v). In addition, a much lower value for the nystose hydrolytic constant was found when purified enzyme was used. In the same direction, Guio et al. (23) modified the original model (20), considering the effect of immobilized glucose isomerase, incorporated to improve FOS conversion. In addition, Detofol et al. (24) proved the accuracy of this approach both on batch and on continuous reactors. According to Vega and Zúniga-Hansen (17), this assumption just partially describes the progress of the reaction because it considers that the same substrate is acting as a donor and acceptor for the fructosyl moiety. However, the active site of fructosyltransferases contains a pocket that accommodates a single sucrose molecule in the substrate-bound structure. Therefore, they proposed a mathematical model based on a mechanism in which sucrose and FOS interact with the enzyme species applying multi-response non-linear regression. This concept was also developed by Khandekar et al. (25) who presented a five-step, ten-parameter kinetic model based on the Michaelis-Menten concept but including the step of binding

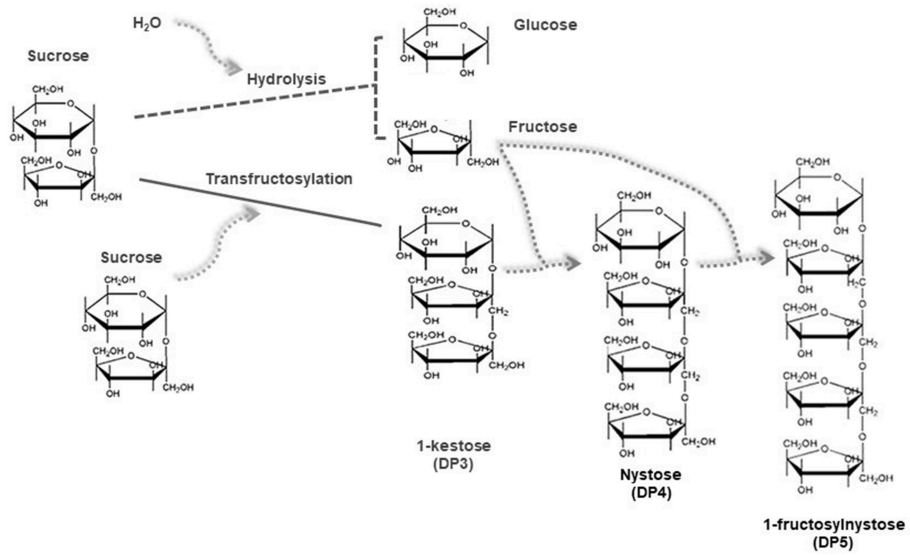

FIGURE 1 | Simplified scheme for the enzymatic synthesis of FOS through transfructosylation reactions.

sites of the enzyme, with sucrose as substrate and glucose as an inhibitor, and also the occurrence of FOS hydrolysis. These contributions regarding the mathematical models explaining the mechanisms involved in the synthesis of FOS were the most important ones reported in the last years. It is worth to mention that the more accurate the model, the better its capacity to explain technological aspects, namely the improving of reaction conditions or yield of the products.

The composition of the obtained FOS can be modulated by adjusting different parameters, namely substrate concentration, enzyme source, time, temperature and pH, all of them interacting with each other. Therefore, when searching for the optimal value of one of these parameters, the values of the other ones must also be taken into account. **Table 1** summarizes several research works on this field, specifying enzyme source, reaction temperature, pH, time, substrate concentration, amount of enzyme, and the resulting FOS yield.

Regarding substrate concentration, in general terms, higher initial concentration of sucrose (i.e., >40%), enhances the production of shorter FOS [i.e., 1-kestose (DP3) and nystose (DP4)], with low production of glucose. On the contrary, lower concentrations of sucrose lead to the production of larger FOS [i.e., DP5 and DP6] with a higher production of glucose (10). Some authors claimed that using very high initial concentration of sucrose (85% w/v) is a technological strategy for the production of commercial syrups. This way, the final evaporation step is simplified (43). The modulation of the synthesis regarding initial substrate concentration is important to accurately obtain FOS mixtures with better prebiotic effects, taking into account that the shorter the chain length the greater the prebiotic effect (44). As stated before, the synthesis of FOS occurs through consecutive sets of disproportionation reactions in which the FOS synthesized in the first steps act as fructosyl donors and acceptors leading simultaneously to the production of FOS with DP immediately higher. Consequently, when the maximum conversion of DP_n is reached, it is followed by a decrease of DP_n, leading to an increase in DP_{n+1}. Taking this into account, it is crucial to know these kinetic parameters (maximum conversion and time at which is reached) to modulate the product composition, not forgetting their dependence on other reaction conditions (pH, temperature, enzyme source, enzyme concentration). In this sense, many authors have studied the effect of substrate concentration on the enzymatic synthesis of FOS, under different conditions (10, 16, 28, 29, 31, 40, 45). However, only few authors investigated the effect of more than one parameter at the same time. For example, Nemukula et al. (30) proposed a joint analysis of the effect of sucrose concentration, enzyme concentration, reaction time, temperature and pH for obtaining the maximal FOS yield, using response surface methodology. In line with such study, Vega and Zúniga-Hansen (15) studied the interaction of sucrose concentration, temperature and enzyme concentration on FOS, DP3, and volumetric yield. These approaches enabled to determine the most appropriate cost-effective condition to operate (operation temperature 50°C, pH 5.5, 6.6 TU/mL of enzyme, and 71% w/v of initial sucrose concentration), which enabled obtaining 63.8% of FOS yield (short chain FOS grams per 100 g of initial sucrose).

Concerning the enzyme source, all enzymes used for producing FOS (both by synthesis and by hydrolysis) generally belong to the glycoside hydrolases family (GH) and are either included into the GH32 or GH68 families (CAZy classification) (46). Particularly, enzymes with fructosyltransferase activity can be found in plants, yeasts and molds (GH32) and in bacteria (GH68) (47). Most commercial enzyme preparations have both fructosyltransferase and hydrolase activities; this

TABLE 1 | Different enzyme sources, conditions performed, and yields for the synthesis of FOS.

Enzyme source	T (°C)	pH	Sucrose (g/100 ml)	Enzyme amount	Reaction time (h)	$Y^{a}_{FOS\ max}$ (gFOS/100 g sucrose)[a]	Observation	References
COMMERCIAL								
Rohapect CM (AB Enzymes GmbH)	45–60	5.5–6.5	53–72%	3.4–7.4 UT/mL[d]	3 and 5	41–64%		(15)
Viscozyme L (Blumos SA)	55	5.5	10–60%	56 FU/mL[e]	6	65–85%		(10)
25 enzyme preparations from fungal strains	45–60	4.5–6	40–80%	4.2–15 UT/mL[d]	6	59–64%	5 enzymes selected.	(16)
Viscozyme L (Novozyme)	50	5.5	60%	1,230 UT/g[d1]	2.5 (50 batchs)	40–6%	purified immobilized enzyme.	(26)
Pectinex Ultra SP-L & Rapidase TF (Novozyme)	60	5.6	63%	0.3 U/mL[f]	144	62%[h]	immobilized enzyme.	(27)
MICROORGANISM								
A. japonicus TIT-K J1	37	5.4	10, 30, 50%	0.2, 0.56, 0.96 U/mL[f]	24	65–68%		(28)
A. japonicus	50	5	45–70%	5.75 g cell/100 mL	4	51–59%		(29)
A. aculeatus	50-70	4.8–6.4	20–60%	20–100 U/mL[f]	4-24	55% DP3[h]; 43%DP4[h]		(30)
A. niger	55	6	10,30,60%	0.66 U/mL[f]	88	55-45%[h]		(31)
S. cerevisiae (invertase)	40–55	5.5	21–85%	0.5–8.0 U/mL[f]	8	10% (d.b.)[c]		(25)
Rhodotorula sp.	50	4.5	50–70%	5 UTF/mL	96	50–58%		(22)
Rhodotorula sp.	48	6	50%	0.022 U/mL[f]	48-56-72-96	44–60%	immobilized and free enzyme.	(24)
Aureobasidium sp. ATCC 20524	30	5.5	40%	270 U/g [g]	20 mL/h (26 days)	1,512 g DP3	immobilized enzyme; continuous reactor.	(32)
Cryptococcus sp.	50	4.5	50%	1 FTA/mL	48	34%		(33)
A. niger IMI 303386	39	6.5	50%	0.4 U/mL[f]	72	62%[h]		(34)
B. subtilis natto CCT 7712	35-55	7.7	20-40%[i]	n.i.[b]	12–36	388 mg/mL[j]		(35)
Levansucrase SacB of *B. subtilis*	37	6	9%[k]	1.47 U/mL[f]	24	54%		(36)
A. niger AS0023	50	5.8	50%	5×10^6 KU	5	62%[h]		(37)
A. foetidus	40–70	3–7	30%[l]	n.i.[b]	12	29–48%		(38)
Arthrohacter sp. K-I	40	6.5	10%	3.4 U/mL[f]	5–20	n.i.[b]	[m]	(39)
A. niger; *A. awamori*, *S. cerevisiae*	40	5	50%	6 U/g sucrose[f]	8–72	50–37%[h]		(40)
A. niger, A. pullulans.	50–65	4–8	70%	1:9 (w/w) cell:sucrose	8	35–38%		(41)
A. oryzae	55	5–6	60%	0.14 (v/v) Culture/sucrose	4–24	55%		(42)
A. oryzae	60	5.5	75%	275 U/g sucrose[f]	7	57%		(23)

[a] Y_{FOSmax}: *Maximal yield of FOS;*
[b] *n.i. not informed;*
[c] *d.b.: dry basis;*
[d] *UT/mL: transfructosylation activity/ mL of reaction volume;*
[d1] *UT/g: transfructosylation activity/g of dry support;*
[e] *FU/mL: fructosyltransferase units/ mL of reaction volume;*
[f] *U/mL: One unit of enzyme activity; the amount that produce l*μ*mol of reduced sugar per minute/ mL of reaction volume;*
[g] *U/g: One unit of enzyme activity; the amount that produce l*μ*mol of reduced sugar per minute/g of dry support;*
[h] *Informed yield: weight percentages of total sugar;*
[i] *Substrates: sucrose, sugarcane molasses and sugarcane juice;*
[j] *Yield informed as amount of DP4 produced;*
[k] *Substrates: sucrose and sucrose analogs;*
[l] *Substrates: maltose or sucrose;*
[m] *Analysis based on transfructosylated products and acceptor specificity.*

combination gives them advantages over specific enzymes, such as low price, versatility and high stability under reaction industrial processes conditions, but the disadvantage of non-probiotic monosaccharides (i.e., glucose and fructose) being also produced as result of the enzymatic reaction (**Table 1**). Therefore, preparations with high transfructosylase activity are preferred for the synthesis of short chain FOS. Vega and Zúniga-Hansen (2012) (16) studied twenty-five commercial enzyme preparations from the global market (Europe, USA and South America) to obtain short chain FOS from sucrose, weighing up both transfructosylation activity and transferase/hydrolase ratio. As an example, the enzyme *Viscozyme L* from *Aspergillus aculeatus* (Novozyme, Denmark) simultaneously has high transfructosylation activity and high transferase/hydrolase ratio (16, 26, 48) (**Table 1**). The enzymatic transfructosylation of sucrose with bacterial or fungal fructosyltransferases (23, 33, 42, 49) or fungal β-fructofuranosidases (32, 34) have also shown promissory results. In this regard, using extracellular β-fructofuranosidases from different fungus, together with cultivation with *Picchia pastoris* increases the production of FOS DP3 (26.47%) and DP4 (57.98%) (50). Other reports describe the capacity of *Bacillus subtilis* natto CCT 7712 to produce high amounts of DP5 (nystose) from low-cost substrates, such as sucrose, sugarcane molasses, and sugarcane juice (35). Each type of enzyme was tested for FOS production under different conditions and the results and main particular observations are presented in (**Table 1**).

Besides the enzyme characteristics, biocatalysts can be free (10, 11, 15–17, 23, 36) or immobilized (26, 27, 51) in the reaction medium. Immobilization consists on turning the enzyme into a physically confined form in a defined region, blocking its mobility but maintaining its catalytic activity. Many authors reported a higher catalytic efficiency of enzyme membrane reactors employing free enzymes for relatively long periods (52). Although the enzymatic production of FOS using immobilized enzymes may not work optimally due to limited substrate or product mass transfer to and from the enzymes, it is a relatively new alternative, whose main advantage is offering the possibility of re-using the enzyme. This great advantage denotes the need of further research to overcome the mentioned inconvenients regarding immobilized enzymes.

Optimal pH and temperature strongly depend on the enzyme source. As it is shown in **Table 1**, the reaction can be performed in a widely pH range of (3–7), and the temperature can vary from (35–70°C). Nevertheless, in general terms there can be mentioned that more bounded ranges of optimal pH and temperature can be defined by gathering together more than one type of enzyme. In this respect, a large number of reports have placed the optimum pH and temperature for activity of fructosyltransferase between 4.5–6.5 and 40–60°C, respectively (30, 53, 54) (**Table 1**). These two parameters fundamentally affect reaction rates. In this sense, Vega et al. (16) who studied the effect of temperature reaction in a range of 45–60°C, found that the increase of reaction temperature causes an increase in the reaction rate. A similar behavior has been reported by other authors (15, 41, 55). It is important to mention that over 60°C the enzyme could present thermal damage and its activity decreases considerably (26). Regarding pH, it has strong impact in the ionization state of the constituent amino acids, thus affecting the enzyme's primary and secondary structure and consequently its activity (56). A pH of around 5.5 was reported to be optimal for fructosyltransferase production in *Penicillium purpurogenum* (57), *Aureobasidium pullulans* (58), and *Syncephalastrum racemosum* Cohn (59).

In general, the synthesis of FOS yields about 60% FOS, under the form of syrup. Most commercial FOS products are mixtures containing different amounts of FOS, products with 55–99% of purity. The presence of glucose (and residual sucrose) obtained as secondary product of reaction decreases the prebiotic effect of the mixtures, increasing their caloric and cariogenic value, and thus preventing their incorporation into health, dietetic and diabetic foods (60). To enhance the purity of FOS, mono and disaccharides can be removed. One option is the continuous removal of glucose and residual sucrose during the synthesis using enzymes and membrane reactors (61, 62). Another option is the purification process after the synthesis. There are many strategies in the research background of FOS production, but generally both activated charcoal adsorption and enzymatic methods are the most extensively used.

Purification of FOS using activated charcoal consists on the adsorption of sugars onto the activated charcoal, in a reversible process. As activated charcoal is non-polar or hydrophobic, sugars are adsorbed according to their hydrophobic character due to van der Walls forces, which is directly related to their molecular weight (the higher the molecular weight, the more CH groups and the more hydrophobic the sugar is). Hence, FOS are more strongly adsorbed than mono and disaccharides, enabling their separation (60). In practice, purification involves the filling of columns with activated charcoal (sorbent) and the re-circulation of the obtained syrups until an equilibrium between the sorbent and the moving phase is reached. After that, the non-adsorbed sugars are removed by circulating milli-Q pure water through the column. Finally, the retained oligosaccharides are recovered by elution with different ethanol gradients (40, 63–65). The products of elution are also syrups that can be concentrated and even dehydrated to obtain powders (11). The mechanisms involved in the purification of oligosaccharides using activated charcoal are determined by their molecular interactions. Packer et al. (66) deeply analyzed the efficiency of using graphitized carbon to separate oligosaccharides or their derivatives (hydrazones and alditols) released from glycoproteins from solutions containing salts (of hydroxide, acetate, phosphate), detergents (sodium dodecyl sulfate and Triton X-100), and proteins (enzymes, glycoproteins). Reagents such as hydrazine or sodium borohydride were reported to release oligosaccharides. Fractionation of neutral and acidic oligosaccharides, which are sialylated, sulfated or phosphorylated, is also possible by elution with water-acetonitrile mixtures. Although the use of such desorbents might be useful for FOS purification, the alimentary use of FOS must not be forgotten. Therefore, when FOS are to be purified, only GRAS (Generally recognized as safe) products are allowed.

Enzymatic oxidation of glucose is an alternative to purify the synthesized FOS. This method is as efficient as the former,

but much easier to scale-up. The glucose can be oxidized using glucose-oxidase as biocatalyst, producing gluconic acid, which can be precipitated by the addition of $Ca(OH)_2$. This calcium gluconate can be also used as source of calcium. This way, the glucose generated during the enzymatic synthesis of FOS can be transformed into other products of high added value (11). Additionally, if the synthesized FOS are treated with immobilized cells of *Zymomonas mobilis*, glucose, fructose and sucrose can be simultaneously eliminated (67). Other methods to remove mono and di saccharides from FOS syrup, are membrane technology, mainly ultra and nanofiltration (68–70), and also microbial treatment through the fermentation of glucose, fructose and sucrose to ethanol and carbon dioxide (60, 71, 72). This method involves additional process to treat fermentation products, and depending on the microorganism selected and the raw material used, additional nutrients may be necessary (67).

No matter purification process, the mixture of purified FOS still contains different concentrations of FOS with different DP. As they are usually employed in the formulation of functional foods or in infant formula, purification of each oligosaccharide is not strictly necessary. However, for mechanistic or physiological investigations, the availability of pure FOS with a given DP is necessary. The isolation is possible using preparative HPLC although it is not an easy process, especially for the production at a large scale. Indeed, pure FOS are expensive and are only available for analytical purposes.

FOS Obtained by Hydrolysis of Inulin

In general, the presence of mono and disaccharides in the final product is one of the drawbacks of synthesis of FOS over the hydrolysis from inulin, making the yield and purity of the latter much higher. In this regard, the production of FOS using endo-inulinases yields 81%, compared to the 55% resulting from fructosyltransferases activity (73).

Plant inulin have chains of up to 60 units of fructose, which length, composition and dispersity vary with plant species, life cycle phase, time of harvest and the conditions of extraction and post-extraction. Fresh plant material is always used to extract native inulin, and precautions must be taken to inhibit the plant own inulinase activity and to prevent acid hydrolysis. Even so, the extraction of inulin is always accompanied by the extraction of FOS, sucrose, fructose and glucose in variable amounts. Inulin is soluble in water in moderate extent (about 10% at 20°C), producing a low-viscosity solution. It can form a tridimensional microcrystalline gel network at higher concentrations; this will give a fat-like mouthfeel. Inulin is about 10 times less sweet than sucrose and that sweetness is eliminated when short chain inulin molecules are removed. This process increases the gel-forming capabilities.

Commercially available inulin is currently produced by the industry from two species belonging to Compositae: Jerusalem artichoke (*Helianthus tuberosus*) and chicory *(Cichorium intybus*); however, commercial inulin from dahlia (*Dahlia pinnata*) tubers can also be found (74). Additionally, it can be extracted from the tubers of *Cynara cardunculus* (artichoke) and *Polymnia sonchifolia* (yacon) (75). Agave, garlic and shallots are also potential sources (76). Jerusalem artichoke is one of the most important raw materials for the industrial production of fructose and inulin since it is easy to cultivate, accumulates about 50–70 g/kg of its fresh weight as inulin-type fructans and the crop yield estimate is 5.4 ton/ha (77). However, both inulin contents and degree of polymerization vary extensively with time of harvest (78). This may lead to variation in the composition, something common in natural products, but a possible issue for some applications in which a very precise composition is required.

Inulin may be commercially obtained in different forms: native inulin with an average degree of polymerization (DP) of 10–12, containing short chain inulin fractions (DP 2–10) and high performance inulin (HP) with DP higher than 20. Small inulin oligomers mixture with DP<10 are often designated by oligofructose or short-chain FOS. The long-chain inulin or inulin HP is produced by physical separation techniques.

Mensink et al. (79) revised the origin, physico-chemical properties and DP of commercially available inulins. They highlight that two batches of inulin with the same average DP can have different size distributions and therefore there characteristics can be very different. Inulins with higher DP have lower solubility in water, higher melting temperatures (crystalline inulins) or higher glass transition temperatures (amorphous inulins), higher chemical stability (do not hydrolyse easily), form stronger gels and their aqueous solutions have higher viscosity.

The fructose units of inulin are linked by β-(2→ 1) D-fructosyl-fructose bonds and the chain thus formed is usually terminated with one glucose unit linked through an α-D-glucopyranosyl or α-(1→ 2) bond in the same way as in sucrose. Inulins that show this terminal glucose unit are designated by α-D-glucopyranosyl-[β-D-fructofuranosyl]$_{n-1}$-D-fructofuranosides (FOS or GF_n), while those that lack this glucose unit and are therefore constituted of fructose only are called fructopyranosyl-[α-D-fructofuranosyl]$_{n-1}$-D-fructofuranosides (or inulo-oligosaccharides -IOS or FF_n) (80).

The extraction of inulin and FOS from vegetables is carried out by grinding and solubilization in hot water, with further enzymatic treatment with sucrases (to eliminate the sucrose still present), α-amylase and maltase (for degradation of short chain carbohydrates) (81).

Enzymatic hydrolysis of inulin is the most common procedure, however other methods such as acid hydrolysis and auto-hydrolysis can also be employed for this purpose.

Enzymatic Hydrolysis

There are two types of hydrolytic enzymes that break down inulin, endo- and exo-inulinases. As in October 2018, BRENDA (the free comprehensive enzyme system–www.brenda-enzymes.org) has 58 endo- and 70 exo-inulinases described. These enzymes can be obtained from bacteria, fungi, yeast, and plants, although commercially available products come from the fungus *Aspergillus* spp., in particular *A. niger* (www.brenda-enzymes.org).

Endo-inulinases (E.C.3.2.1.7) are enzymes capable of cleaving linkages between fructosyl moieties/residues within the fructan chain. They have been widely used for the production of FOS, especially since the commercial inulinase form is the endolytic type (**Figure 2**). These enzymes can also be used to determine

the overall content of inulin and FOS in plants and foodstuffs by measuring the amount of fructose, glucose, and sucrose before and after the enzymatic hydrolysis (76).

Exo-inulinases hydrolyze terminal, non-reducing 2,1-linked and 2,6-linked β-D-fructofuranose residues in inulin, levan and sucrose releasing β-D-fructose. Most exo-inulinases are capable of hydrolyzing inulin in a very effective way, producing fructose in yields as high as 90–95%, so they are used mainly for the production of ultra-high-fructose syrup.

The enzyme source can dictate the outcome of the hydrolysis: amount and type of products generated. For instance, the production of FOS using endo-inulinases from *Xanthomonas oryzae* No. 5 (82) results in FOS with DP≥5 as the major compounds, while the same enzyme from *Pseudomonas* sp. No. 65 (83) produces mainly DP2 (inulinobiose) and DP3.

In an assay with endo-inulase from *Pseudomonas* sp. No. 65, DP2 (inulobiose) and DP3 FOS were the main products (31 and 23%, respectively) when using pure inulin from chicory and, with raw chicory extract, the hydrolysate consisted of 19% DP2, 19% DP3, 14% DP4, and 19%>DP5, with fructose, glucose, and sucrose being detected in both cases. Additionally, dual systems with different endo-inulinases can also be used for the production of FOS from inulin, accordingly to the user's needs (84).

The high cost of the enzymes increases the overall cost of this process (85). This has been partially overcome through genetic engineering and molecular biology techniques, many modified enzymes with enhanced properties compared to their natural counterparts have been obtained (86–88).

Endo-inulinase genes from microbial species have been successfully cloned in another and the expressed enzyme used for the hydrolysis of inulin with noteworthy results (89). It is possible to design a system suited to the user's needs, such as high expression, intra or extracellular enzymatic production and thermoresistance. Recently, an endo-inulinase encoding gene was cloned and transfected into *Baccillus subtilis* WB800-R and the enzyme produced was used for the hydrolysis of inulin resulting in yield of around 69 g/L of FOS, mainly DP3, 4 and 5, in the crude extract, with a conversion rate of pure inulin into FOS of 75% (90).

Wang et al. (91) reported a simple and highly efficient one-step bioprocess for production of high-content FOS from inulin by yeast fermentation, using a recombinant yeast strain JZHSTSC, in which a heterologous endo-inulinase gene was expressed and the inherent invertase gene SUC2 was disrupted. This yeast simultaneously hydrolyzed inulin into FOS by secretion of endo-inulinases and removed mono-sugars by assimilation, resulting a product with high purity of FOS (∼90%).

In a similar process, but in a two-step way, Han et al. (92) achieved similar results by using a recombinant *Yarrowia lipolytica* strain Enop56, in which an optimized endo-inulinase gene from *Aspergillus niger* was overexpressed. The hydrolysis in these conditions lead to the formation of FOSs with DP 3–5 as major products and to <5% of mono- and disaccharides (non-prebiotic). As before, large amounts of FFn oligosaccharides were obtained. Since both GFn and FFn oligosaccharides show identical functional and physiological properties, this is not a disadvantage (93).

Several studies of cloning and modification have been performed on fungal inulinases in order to improve efficiency, achieving yields up to 90% of oligofructose with degrees of polymerization between 3 and 6 (94).

Acid Hydrolysis

Glibowski and Bukowska (95) found out that heating 5% inulin solution in a strong acidic environment (pH 1–3) caused intensive hydrolysis, even mild temperatures (40°C) which somehow contradicts the notion that inulin is not digested by the human gastrointestinal tract.

It has been reported that both fructose and fructooligosaccharides can be produced from inulin by chemical hydrolysis (pH 1–2 at 80–100°C), but fructose degrades easily at low pH resulting in the formation of di-fructose anhydride, a colored byproduct with almost no sweetening capacity, and hydroxymethylfurfural, a known by-product and inhibitor for fermentative organisms.

Acid hydrolysis becomes relevant in the obtaining of FOS from agave, since the amount of fructan accumulated in the mature plants [13–17% (w/w) fresh weight] is similar to what is found in the current source of inulin, chicory [15.2–20.5% (w/w) fresh weight]. The main difference resides on the structure of the fructose polymers: while in chicory inulin fructose molecules are joined through β(2–1) linkages in linear chains, fructans present in agave, especially in *Agave tequilana*, have a relevant content of β(2–6) linkages resulting in branching fructose molecules (levan type fructans) in chains with DP 3–29. Due to their complex structures, commercial *endo*-inulinases have little hydrolytic activity over these polymers, while specific endo-levanases are difficult to obtain and fructanases, combining *endo* and exo-inulinase activities, lead fructose as the main hydrolysis product, even at low conversions. Avila-Fernadez et al. (96) used a limited acid hydrolysis by HCl and cation exchange resins for the production of FOS from agave fructans; the reaction need to be controlled to prevent hydrolysis to fructose. β-(2,6)-FOS were prepared from microbial high-molecular-mass levan by acid hydrolysis and refined by cation-exchange chromatography, resulting in oligosaccharides with a DP within 2 and 20 and the same β-(2,6) linkage type. The long-chain β-(2,6)-FOS were more resistant against acid or enzymatic hydrolysis than the short-chain β-(2,6)-FOS.

Hence, acid hydrolysis is suitable when the aim is the production of fructose syrups as an alternative to exo-inulinase hydrolysis or for bioethanol production from biomass (97).

Autohydrolysis

Long-term storage provides adequate conditions for the chemical breakdown of inulin and FOS. This is also the reason why older plants typically have lower inulin contents than younger ones: plants also contain enzymes that can hydrolyze inulin. The main effects are the shortening of the FOS chains and eventually the production of free sugars, that is, glucose, fructose and sucrose.

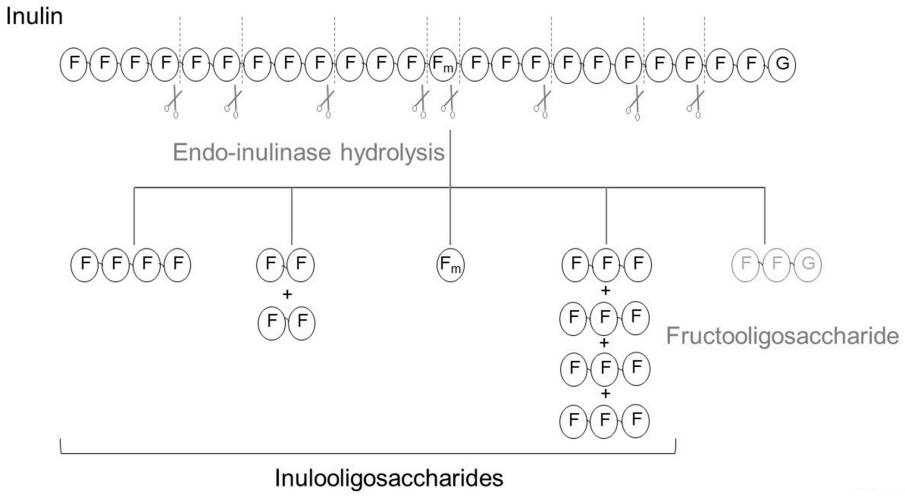

FIGURE 2 | Scheme of the hydrolysis of inulin using endo-inulinase as biocatalyst. Each initial GFn yields one fructooligosaccharide and several inulo-oligosaccharides.

Extracted inulins may contain a large amount of sugars (mono-, di- and small oligosaccharides) (84). Typically, extraction is done by boiling the cleaned and cut or ground up tubers, or other inulin containing plant part, in water. Process conditions (pH, water–root ratio, boiling time, etc.) affect the DP of the produced inulin. Higher oligomers are more hydrolyzed than the lower oligomers, since they have a relatively high content of fructosyl end chains.

The isolation of those small oligosaccharides, which will have a glucosyl end and are thus similar to FOS obtained by synthesis, can be an interesting approach.

Cho et al. (84) found 38% of FOS (DP3 to >5) in the initial carbohydrate composition of chicory juice, together with 33% inulin and 27% mono and disaccharides. Precipitation of inulin and removal of mono and disaccharides would lead FOS as the main product.

Other Species Should be Considered as Direct Sources of Oligosaccharides

Benkeblia et al. (98) extracted FOS (DP3 to DP12) from onions in average amounts of 270 mg/g together with free mono and disaccharides in amounts of 450 mg/g; only the fraction of DP5-DP12 degraded with time at 20°C. Shiomi et al. (99) revised the metabolism of FOS in onions, concluding that the maximum amounts are found during dormancy, after the activity of fructosyltransferases during bulbing and before the extensive activity of exo-hydrolases that takes places during sprouting.

Yacon (*Smallanthus sonchifolius* Poepp. and Endl.) is a root crop native to the Andean region, but has also been cultivated in other regions. Yacon tubers are traditionally consumed as fresh fruit due to their crunchy texture and high juice contents, having a moderate sweet taste. Saccharides compose up to 80% of the total dry matter content of yacon tubers, with a large dependence on cultivar. These saccharides consist of fructose, glucose, sucrose and FOS, which are usually as their dominant group of saccharides (100).

Campos et al. (101) studied 35 different yacon accessions and found that the content of reducing sugars (RS), sucrose (S) and FOS based on dry matter vary wildly depending on accession. The highest FOS contents found was 65.0 g FOS/100 g DM. The content of RS in yacon accessions was inversely correlated to the FOS content.

Sumiyanto et al. (102) analyzed the fructans content in tuberous roots of yacon and found values between 70 and 80% of the dry weight during the harvest period of October-December and very little variation in the amount of other solids over this period of time.

The fructooligosaccharides in yacon represent mainly oligosaccharides from DP3 to DP10 with terminal sucrose (inulin-type fructooligosaccharides) (103). Regarding other nutrients of yacon, many studies reported that it contains low protein, lipid and ash content, thus making this tuber a potential source of FOS.

The large variations in mono and disaccharide content may be due to the accession, edaphoclimatic conditions during growth of yacon, and particularly the post-harvest procedures. A common postharvest strategy consisting on exposing the tubers to direct sunlight in order to increase their sweetness will cause the breakdown of FOS to FOS with lower DP and/or free fructose and glucose. Processes such as drying will also modify the profile of carbohydrate content of yacon tubers (104).

A derivative of yacon that is industrially available is yacon syrup, produced by juicing the fresh roots, filtering and concentrating by evaporation of water (105). Since the water contents is diminished to about 20%, the syrup can be stored for several months without significant reduction of FOS content or significant depolymerization (106).

The enzymatic hydrolysis of inulin generally produces chains longer than DP5, with a lower prebiotic activity than those produced synthetically (DP3-5). Depending on the application of the generated FOS, these points should be considered (90). There is the possibility of making use of the action of

exo-inulinases (E.C. 3.2.1.80), which remove frutosyl residues from the non-reducing end of the inulin molecule, thus shortening the chain, but these also hydrolyze sucrose and raffinose, thus produces a mixture containing high amounts of free glucose and fructose, since this enzyme is able to hydrolyze the glycosidic bond α-(1,2), which connects glucose to the main inulin chain, so additional steps of purification are also needed.

The major drawback of inulin as a source of FOS is the fact that it is not a single structure. The chemical structure of fructans vary widely depending on the species. For example, as mentioned before, inulin from some plants has a 2,1-linked -D-fructosyl back bone with 2,6-linked -D-fructosyl side chains in variable percentage, as in garlic and *Agave tequilana* (96), while other have only linear chains. Degree of polymerization differences are another issue: inulin from chicory (*Cichorium intybus*) has a much lower DP (about 20) than inulin obtained from globe thistle (*Echinops ritro*) with mean DP 30 or global artichokes (*Cynara scolymus*) with mean DP65.

The species mentioned in this section also contain their own inulinases, which is the major drawback for inulin recovery. Leroy et al. found that throughout the period of artichoke storage, a decrease in inulin content and mean DP occurs, owing to its *in natura* depolymerization (107). *A. tequilana* was investigated as potential inulin source, the youngest plants exhibited the highest levels of free monosaccharides and low molecular weight fructans with potential application as prebiotics, while the DP reached a maximum of 3–30 in 4-year-old plants and then decreased to 4–24 in the oldest (>6 years) ones (108).

Another important issue is the need to extract and purify inulin from the natural matrix usually requires juices extraction and a succession of freezing, thawing and (ultra)centrifugation in order to remove low DP and other contaminants. Filtrates are deionized by passing through strong anionic and cationic resin exchangers, before a final step of freezing/thawing/centrifugation. As a result, very pure inulin (>98% purity) can be obtained. Depending on the application, this process, albeit tedious and costly, can be very effective specially to obtain FFn oligosaccharides from long chain inulins, since each resulting molecule contains a terminal glucose (GFn) for several FFn oligomers after high purity endo-inulinase hydrolysis. The general mode of endo-inulinase action is that the hydrolytic activity for inulin increases with the degree of polymerization of fructosyl residues (73). For global artichoke, F_3 and F_4 were the main fructose polymers (80).

However, Cho et al. (82) carried out IOS production from chicory root juice, using endoinulinase from *Xanthomonas oryzae* No. 5, and compared with FOS from pure inulin. From their results, hydrolysis with endo-inulinase over the extract does not affect ≤DP4, mainly converting inulin in DP5 and >DP5. So, if the aim is the use of FOS, the initial purification on inulin is not necessary and the removal of mono and disaccharides could be left as final step.

Physical techniques, such as ultrasound, have also been reported as methods for the production of low molecular weight FOS fragments from Jerusalem artichoke inulin extractions (109). Furthermore, ultrasound extraction of *Flammulina velutipes* polysaccharides has also been reported as a method for production of FOS (110).

GOS

GOS are composed by a variable number of galactose units, within 2 and 10. Similarly to FOS, GOS can be obtained either by synthesis or by extraction and hydrolysis. The type of linkage between units varies according to their origin and obtaining process. Plant based GOS are α-GOS whereas GOS prepared from lactose are β-GOS.

α-GOS are important components of seeds, namely pulses, and show a terminal sucrose unit and the linkage between monosaccharide moieties can be [Gal-α(1→ 6)-Gal], [Gal-α(1→ 4)-Gal], [Gal- α(1→ 3)-Gal] and [Gal-α(1→ 6)-Glu-β(2→ 1)-Fru]. This is called the raffinose family (RFO). Another relevant α-GOS is melibiose, a reducing disaccharide with a linkage (Gal-α(1→ 6)-Glc) (isomer of lactose)

β-GOS, also known as oligogalactosyllactose, oligogalactose, oligolactose, transgalactosylated oligosaccbaride, and transgalacto-oligosaccbaride, show a terminal glucose unit and the galactose units are linked mostly by β(1→ 4) and β(1→ 6) bonds (111–113).

Although tri- to hexa-saccharides, with 2 to 5 galactose units (DP3-6), tend to be the main components of GOS-containing products, disaccharides (DP2) consisting of galactose and glucose with β-glycoside bonds such as [Gal-β(1→ 6)-Glc], [Gal-β(1→ 6)-Gal], [Gal-β(1→ 4)-Gal] or [Gal-β(1→ 3)-Gal] which are different from lactose, [Gal-β(1→ 4)-Glc], are also present and defined as GOS since they have physiological characteristics are similar to longer chains.

The prebiotic effect of α- and β-GOS is mainly associated to tri and tetrasaccharides (DP3 and DP4, respectively) (114–116).

GOS Obtained by Enzymatic Synthesis

GOS can be commercially synthesized from lactose through transgalactosylation reactions, using β-galactosidases (EC 3.2.1.23) as biocatalysts (117). The main companies leading GOS market are Yakult Honsha Co Ltd. in Japan with Oligomate 55 syrup and Oligomate 55P powder (both with 55% dry matter of oligosaccharides), and TOS-100, a purified powder containing 99% oligosaccharides (118). Also, Friesland Foods Domo in The Netherlands commercializes TOS-syrup (75% w/v content of GOS) and Vivinal GOS syrup (with 75% w/v of solids which 59% are GOS (4).

α-GOS can also be produced by transgalactosylation reactions of α-galactosidase (α-Gal) or by conversion of raffinose family oligosaccharides by levansucrase. However, there is very little data on transgalactosylation reactions of α-Gal (119, 120), and therefore, all the discussion will be based on the better known β-GOS production.

β-galactosidases from fungi of the genus *Aspergillus* and yeasts of the genus *Kluyveromyces, Rhodotorula, Bullera singularis* and *Sterigmatomyces*, as well as bacteria of the genus *Lactobacillus* or *Bacillus* are generally used as biocatalysts for the industrial synthesis of GOS (121, 122) both for food and pharmaceutical applications (123). These enzymes are widely known for

their glycoside hydrolase activity, leading to the cleavage of β-galactosides into monosaccharides. However, in certain conditions, they can be used as biocatalysts for the synthesis of GOS. Indeed, β-galactosidases identify different types of glucose-glucose bonds [i.e., β(1→ 2), β(1→ 3), β(1→ 4)], as well as β(1→ 6) and β(1→ 3) glucose-galactose bonds, and catalyze the transfer of a galactose moiety from a β-galactoside to an acceptor containing a hydroxyl group. The accepted mechanism for the enzymatic catalysis involves two steps (**Figure 3**):

i- The formation of an enzyme–galactosyl complex, with simultaneous liberation of glucose;
ii- The transfer of the enzyme–galactosyl complex to a nucleophilic acceptor containing a hydroxyl group. If the nucleophilic acceptor is water, galactose is obtained as a product (hydrolysis reaction) (**Figure 3**) (124). If the nucleophilic acceptor is another sugar, di, tri or higher DP GOS are produced (**Figure 3**). The mechanism has been mathematically described by many kinetic models. Boon et al. (125) reported that the best approach for describing GOS synthesis by β-galactosidases is a kinetic model that considers lactose hydrolysis and oligosaccharide synthesis, so there must be taken into account that water or lactose can attack the galactosyl-enzyme complex, and also it must be included glucose inhibition. From a mathematical viewpoint, the problem can be raised by integrated rate equations and fitted by non-linear regression at different concentrations of substrate (126) so each parameter can be estimated separately and independent of the initial lactose concentration (127).

High concentrations of lactose compete with water for the transfer of galactosyl moieties (ii). Therefore, under these conditions β-galactosidases catalyze the formation of GOS (128). On the contrary, lower concentrations of lactose promote lactose hydrolysis rates to glucose and galactose (112). To stimulate the synthesis of GOS, two main approaches are used: the equilibrium approach and the kinetic approach. Both approaches tend to favor transgalactosylation over hydrolysis, the former through high substrate concentration (less water available in the medium) and the adequate enzyme/substrate ratio, depending on the enzyme source (122) and the later through enhancing the kinetic variables that promotes the most favorable rate of product formation (129).

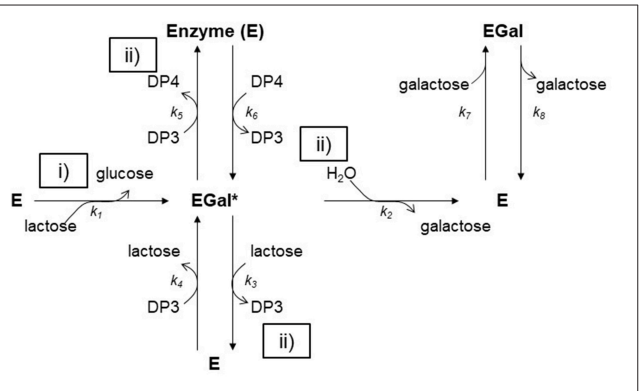

FIGURE 3 | Reaction mechanism for the hydrolysis and transglycosylation of lactose by β-galactosidase. (i) The lactose molecule on the active site of the enzyme forms an acyl-enzyme complex with liberation of glucose; (ii) The enzyme-galactose complex, can react with carbohydrate molecules.

β-galactosidases are the most frequent catalysts used in the synthesis of GOS, although their main application is the hydrolysis of lactose to generate products suitable for lactose allergic people (129). Different species possess different specificities for building glycosidic linkages and therefore produce different GOS mixtures. For example, the β-galactosidase from *K. lactis* produces predominantly β-(1→ 6)-linked GOS, the β-galactosidase from *Aspergillus oryzae* produces mainly β-(1→ 3) and β-(1→ 6) linkages (130), *Bacillus circulans* β-galactosidase forms mainly β-(1→ 4)-linked GOS (131), whereas β-galactosidases from *Lactobacillus* spp. preferably form β-(1→ 6) and β-(1→ 3) linkages in transgalactosylation mode (132, 133). Another important factor regarding enzyme source is the maximum GOS yield and the lactose conversion, that is, the percentage of initial lactose that is consumed during the synthesis. This latter is a very important factor because it has very important nutritional and technological consequences (both the hydrolysis -monosaccharides- and the synthesis products -GOS- are much more soluble than lactose, thus it is possible to go from a suspension to a syrup during the enzymatic reaction). The decrease in the lactose concentration is desirable in people with lactose intolerance. Just to mention some examples, β-galactosidase from *Aspergillus oryzae*, yields 28% of GOS with a lactose conversion of 58% (112), β-galactosidase from *Bacillus circulans* yields 54% GOS (134), and β-galactosidase from the thermophilic archaeon *Thermus caldophilus*, 75% GOS with 50% of lactose conversion (118).

Besides the type of enzyme, generally, the reaction conditions (i.e., initial substrate concentration, temperature, pH or presence of inhibitors or activators of the enzyme) affect the enzyme activity (135, 136). For this reason, all these parameters strongly determine the yield and composition of the GOS obtained, as well as the concentration of mono and disaccharides present in the products of reaction. **Table 2** presents a detailed list with enzymes from different origins used for GOS synthesis, together with the respective reaction conditions and yields. As each type of enzyme has different optimal conditions (lactose concentration, pH, time, temperatures), they have to be deeply investigated to achieve the best performance (lactose conversion, yield of GOS) to obtain the desired composition of GOS.

Similar to the synthesis of FOS, the initial concentration of lactose determines the chemical composition of the synthesized GOS, the more concentrated the substrate, the larger the synthesized GOS (129, 142). Some authors (150) claimed that the initial lactose concentration is directly related with the enzyme activity explaining that higher concentrations favor an increase in GOS yield (121, 127, 138). However, Adamczak et al. (129) investigated the effect of different lactose concentrations and commercial enzymes (**Table 2**), concluding that the lowest lactose concentration used (10%) was the one resulting in the greatest GOS yield 13.7% when Ha-Lactase from *Aspergillus oryzae* was employed as a biocatalyst. Besides, although the

TABLE 2 | Different enzyme sources, conditions performed and yields for the synthesis of GOS.

Enzyme source	T(°C)	pH	lactose (g/100 mL)	Enzyme amount	Reaction time (h)	$Y_{GOS\ max}$ (gGOS/100 g lactose)[a]	Observations	References
COMMERCIAL								
Maxilact 2000 (*K.lactis,* DSM); Lactozym 2000L (*K. fragilis*, Novozyme)]; Ha-Lactase (*A. oryzae*, Chr. Hansen)	40	6.5	10, 20, 30%	40 GAU/g[b]	1	14%[h]		(129)
Biolacta FN5 (*B. circulans*, Vitachem)	4–60	6.6	10 and 5% (skim milk)	0.1 pNPG/g[c]	0–32	54%		(134)
Biolactasa-NTL CONC X2 (*B. circulans*, Biocon)	60	6	50%	40 IU/g[d]	5	39%	Free and immobilized enzyme.	(137)
Lactase *(A. Oryzae*, Enzeco Fungal)	40–55	4.5	40–60%	5–300 IU_T/g[e]	10	17–30%		(138)
A. oryzae (Merck), Lactozyme 2600 L (*K. lactis*, Novozymes), strain K12 (*E. coli*, Worthington)	35	4.5–7	30%[i]	50 U/g[b]	12	25%	3 enzymes compared	(139)
E. coli (Sigma-Aldrich)	10–60	5.5–8	2.5–15%	4.5, 9,14 U/mL[f]	24	49%	[j]	(140)
A. oryzae Genencor International	40	4.5–6	5–50%	4.5 g of support	37 mL/h (2 days)	25%[h]	Immobilized enzyme, continuous reactor.	(130)
*A. oryzae (*Enzeco Fungal Lactase)	50	4.5	20%	388, 250,100 IU_T/g[e]	2.5	33–47%	Immobilized enzyme	(141)
A. oryzae and K. lactis (Sigma), *Bacillus sp.* (Taiwan Fructose Co.)	30–50	5	34%	4.5–10 U/g[g]	18	n.i[b]		(142)
MICROORGANISM								
Sulfolobus solfataricus	70–90	5–7	30–60%	1.2–4.8 U/mL[f]	48–60	50–53%		(143)
S. solfataricus and *Pyrococcus. furious*	70	5.5	4.5–17%[i]	1–2 U/mL[f]	200	50%	Continuous and batch reactor.	(144)
Bifidobacteria (BbgIV)	45–65	6.5	43%	10 U/g [g]	24	49–53%[h]	Free and immobilized enzyme.	(145)
Lactobacillus delbrueckii subsp. *bulgaricus*	30-50	6.5	20%	1.5U/mL[f]	5, 8, 12	48.2–49.5%[h]		(133)
Rhodotorula minuta IF0879	60	5	25%	0.24 U/mL[f]	50	39%[h]		(146)
P. acidipropionici and Lactozym®Pure 6500L (Novozymes)	45	6.5	30%[k]	1.3 U/mL[f]	24	24%[h]		(147)
S.thermophilus DSM2 0259	37, 50	6.5	65%[i]	2.7 U/mL[f]	9	50%[h]		(148)
A. oryzae and *C. laurentii* *A. oryzae* and *K. lactis*	55 45	4.5 6.4	20%	1 IU/g [d] 50 IU/g [d]	3-96	33-34% 25%	Combination of enzymes	(149)
L. reuteri	25–37	6, 6.5	13.5, 30, 60%	195 U/g	70	38%		(132)

[a] *Y_{GOSmax}: Maximal yield of GOS;*
[b] *GAU/g: the amount of enzyme which releases 1 μmol of O-nitrophenol per minute/ g lactose;*
[c] *pNPG/g unit of para-nitrophenol galactoside/ g lactose;*
[d] *IU/g: theamount of enzyme producing 1 μmol of O-nitrophenol per minute/g lactose;*
[e] *IU_T/g: the amount of enzyme that catalyzes the transglycosylation of 1 μmol of galactose per minute;*
[f] *U/mL: theamount of enzyme producing 1 μmol of O-nitrophenol per minute/mL substrate solution;*
[g] *U/g: amount of β-galactosidase needed to liberates 1 lmol glucose per min/g lactose;*
[h] *Informed yield: weight percentages of total sugar;*
[i] *Substrate: whey permeate;*
[j] *Glucose and galactose (10 or 50 g/L) were added to evaluate inhibition effect;*
[k] *Substrate: lactose or lactulose.*

solubility of lactose in water is rather low (220 g/L at 25°C (151), this is not a limitation for the synthesis of GOS. Even when suspensions of lactose with constant shaking can be used as a substrate, the employ of thermostable enzymes enables the synthesis at higher temperatures, which also increases the solubility of lactose. Also, Gosling et al. (134) used a commercial enzyme preparation and 5 and 10% w/v lactose as a substrate, achieving a yield of 50% of GOS regarding initial lactose content. In this sense, Petzelbauer et al. (144) achieved high conversions of lactose into GOS by using a thermostable enzyme that allows to operate at 70°C, thus allowing a continuous hydrolysis of lactose (**Table 2**). Moreover, it was reported that when GOS synthesis was carried out with saturated lactose solutions, the specific enzyme productivity increased while maximum yield slightly decreased with temperature (138). When partially dissolved lactose was employed, an increase in temperature produced an increase in both yield and specific productivity (138). In addition, the continuous removal of the synthesized GOS drives the reaction over time to consume different concentrations of lactose (152). At this point, it should be pointed out that in spite of the several attempts to counterbalance the low solubility of lactose, it must not be forgotten that lactose is a very cost-effective substrate and its price is not a limiting factor for the synthesis of GOS. Only when the lactose used as a substrate takes part of a more complex matrix, such as when using milk or whey permeate, the effect of higher temperatures should be especially considered. In such cases, thermostable enzymes are a good strategy to enhance GOS yields, but the increase in reaction temperature during synthesis must be controlled, as Maillard reaction can occur due to the presence of amino side-chains of proteins and sugars (150).

The reaction temperature is directly related with the lactose concentration (lactose solubility, as mentioned before) and the stability of β-galactosidases (stability of enzymes). During the last decades increasingly interest have raised to find thermostable and thermoactive versions of β-galactosidases (153–157). One of the main enzymes used for the synthesis of commercial GOS is BgaD, obtained from *Bacillus circulans*, and used for the synthesis of GOS commercialized as Vivinal (Orafti), BiOligo® (Ingredion) Purimune™ and Yakult Oligomate 55®. The enzyme is stable up to 65°C (optimal temperature *ca.* 60°C), thus enabling high lactose concentrations (**Table 2**). Other thermostable β-galactosidases (recombinant) were studied even at temperatures above 80°C (143, 158). These enzymes showed an increase in reaction yields given that higher temperatures favor higher rates, high lactose solubility, and favorable equilibrium for transgalactosylation reactions (144, 159).

Regarding pH, several studies claimed that the optimal pH for GOS production is in a range of 6–7 (143, 160–163). However, a more certain pH value must be adjusted considering the enzyme source (150) (**Table 2**). In particular, commercial β-galactosidase from *Aspergillus oryzae* is more efficient in acid than in neutral solutions. Nevertheless, Rodrigues Mano et al. (139) confirmed that transgalactosylation activity for this enzyme have a stronger dependence on lactose concentration than on the pH of the solution.

Experimental research outlined that galactose and/or glucose commonly act as inhibitors for many β-galactosidases. Although galactose is recognized to have a greater inhibitory effect than glucose because it directly competes with lactose to form the galactosyl-enzyme complex (150, 153, 164, 165), this issue is quite controversial and strongly depends on the enzymes and reaction conditions. There are reports showing that for some enzymes neither glucose nor galactose are inhibitors (122), some enzymes have only galactose as inhibitor (165), and some others are inhibited by both sugars (158). As galactose is a competitive inhibitor of most of the β-galactosidases (especially in the hydrolysis of lactose), high concentrations of lactose can counterbalance this inhibitory effect (113). On the contrary, galactose is used to enlarge the chains of GOS during the transgalactosylation reaction (113). Glucose was claimed to have a greater inhibitor effect in some cases (125) and to have similar inhibitory effect (166) respect to galactose. In this regard, glucose is an inhibitor of β-galactosidases from *Lactobacillus reuteri* (132), *Sulfobacterium solfataricus* (155), *Thermus* sp. (167), *Kluyveromyces lactis* (168), *Thermus* sp. (169), and *Caldicellulosiruptor saccharollyticus* (158). As the inhibitory effect of glucose mainly occurs during GOS production (113). Therefore, the desirable enzymes are those with low inhibition of lactose hydrolysis by glucose. The inhibitory or activator effects of glucose and galactose are also dependent on the enzyme source and on the concentration of reagents and products (170). Hence, β-galactosidase from *Kluyveromyces fragilis* was reported to be affected by both combined and individual effects of lactose, glucose and galactose. Glucose is an activator at low concentrations of lactose and galactose and an inhibitor at higher concentrations of these sugars. In turn, galactose becomes an activator of the enzyme at high concentrations of glucose and low concentrations of lactose.

The enzyme is one of the major cost factors for the synthesis of commercial GOS. Therefore, immobilization of β-galactosidases deserved great attention in the last decade, as a way to improve their stability, enable their reutilization and facilitate their removal from the reaction medium. All these advantages enhance the yield of GOS in relation to the enzyme concentration (higher g GOS/ IU of enzyme). Immobilization technique requires a carrier that interacts with the enzyme through physical adsorption, entrapment or covalent binding (171, 172). Different parameters define the efficiency of the support, namely mechanical resistance, enzyme interaction, particle size, specific surface area, among others. Regarding mechanical properties of the support, they rather depend on the final configuration of the reactor than on the application. For example, for a fixed-bed reactor, rigidity is a desired characteristic for the support to bear high pressures, thus, silica-based materials, carbon materials, porous glass, and other mineral materials are good choices in this case (173). On the other hand, if the process is carried out in a stirred-tank reactor, flexible materials (i.e., agarose beads, cellulose beads, Lentikats -polyvinyl alcohol polymers shaped like a lens-) are more adequate (174). With respect to enzyme interaction, physical adsorption on different scaffolds (i.e., cellulose, starch, charcoal carbon, diatomeaceus earth,

Shephadex, cotton cloth, chitosan) has the advantage of being cost-effective with little influence on the enzyme conformation (171). Although the weakness of the binding forces represents a disadvantage of these methods, a treatment with glutaraldehyde can stabilize the enzyme adsorption. In what concerns entrapment methods, enzymes are enclosed in polymeric matrices (i.e., alginate beads, carrageenins, polyacrylamide) or in membranes (i.e., nylon, cellulose, polyacrylamide). These methods are simple and mechanically resistant but enzyme desorption is more difficult compared to the physical adsorption, and requires cross-linking (172). Finally, covalent binding scaffolds establish covalent bonds with the functional groups of the enzyme (amino, carboxyl, hydroxyl, and sulfydryl groups), taking care of protecting the active site. They include eggshell, nylon, zeolite, gelatin, and Sephabeads-epoxy for thermo-stable enzymes. Particle size is another factor to consider depending on the operative characteristics of the synthesis. In general, large particles may be retained more easily than small ones, but they may produce preferential ways in column reactors or present diffusional problems given that long pores may decrease the rate of enzyme adsorption. At last, pore size and specific surface area of a porous particulate support are related parameters: in general, the larger the pores, the smaller the specific area. There must be reached a compromise solution considering loading capacity and size of protein/substrates (175).

For β-galactosidases immobilization several scaffolds were analyzed depending on the enzyme source, both in batch or in continuous operations, and reactions were carried out within 37 and 55°C and pH within 3 and 6.5. Enzymes from *A. orizae* were immobilized in covalently bound cotton cloth (130), in activated chitosan (83, 141, 176, 177), in the form of self-supported cross-linked aggregates (178), in amino-epoxy sepabeads (141), in glyoxyl agarose (137, 141), in magnetic polysiloxane–polyvinyl alcohol beads (127), in magnetic particles coated with polyaniline (179) in magnetite nanoparticles (176). In turn, β-galactosidases from *Bacillus circulans* were immobilized in epoxy-EupergitC (180, 181), in microporous polyvinylidene fluoride or polyvinylidene difluoride (PDVF) membrane (182), or in activated agarose (137). Finally, enzymes from bifidobacteria were immobilized in DEAE-cellulose (145), Q-Sepharose (183), amino-ethyl agarose (184). Enzymes from *Kluyveromyces lactis* were immobilized in glutaraldehyde activated chitosan (185) or in the form of whole permeabilized cells containing the enzyme (186, 187) and enzymes from lactobacilli, in microcrystalline cellulose (188), in PVC silica sheets, active carbon, porous glass beads (189). Among all these strategies, the immobilization in activated agarose (137, 141), in activated chitosan (176, 177), in magnetic polysiloxane–polyvinyl alcohol beads (127), in the form of self-supported cross-linked aggregates (178), and in the form of whole permeabilized cells containing β-galactosidase (186) appear as the most promising ones in terms of maximum yield of GOS and highest productivity (gGOS per liter per hour) (113).

Beyond all these reaction parameters and immobilizing strategies that can be modulated to enhance enzyme activity, the yield of GOS resulting from the enzymatic reactions is in general, relatively low. These can be easy deduced by comparing **Table 1** with **Table 2**. The maximum GOS yield regarding the initial lactose concentration rounds 50% (**Table 2**) while that of FOS regarding initial sucrose concentration often overcomes 60% (**Table 1**). Moreover, their composition, both in type of linkage and molecular size distribution strongly depends on the enzyme used (190). Glucose, galactose and lactose that did not react are the main secondary products of the enzymatic reactions. Likewise in the synthesis of FOS, they shall be removed.

To this aim, similar chromatographic methods such as size exclusion chromatography (191–193) and charcoal-celite chromatography (150, 194) have been proposed.

Selective fermentation is another strategy to remove monosaccharides (135, 142, 194, 195). It basically consists on an anaerobic glycolysis by yeasts, in which the monosaccharides are converted into ethanol and CO_2 (72). This method has the advantage that can be performed directly during the synthesis, and the disadvantage that removal of yeast cells and ethanol are necessary to obtain the purified GOS (135).

Another technology available for GOS purification is ultrafiltration (122), a process where fluid containing enzyme and product flow at a high rate across a membrane surface at a certain fluid pressure. Commonly, membrane pore-size is designed to retain the enzyme while smaller molecules (GOS) are permeated (13). Given that ultrafiltration usually does not ensure the complete elimination of monosaccharides (low molecular weight), nanofiltration appears to be a potential industrially scalable method for purification and concentration of oligosaccharide mixtures (196–199).

Additionally, *in situ* adsorption or precipitation of the undesired sugars (194, 200) are other alternatives for the removal of glucose and lactose. More recently, using immobilized enzymes enabled the simultaneous synthesis of GOS and elimination of mono and disaccharides (201). To this aim, β-galactosidase from *Aspergillus oryzae* was immobilized in glyoxyl-agarose of different particle sizes (fine and macro). At higher lactose concentrations, the hydrolytic potential of the enzyme was of 16 and 30%, and the ratio of transgalactosylation to total reaction, 70–84%.

Obtaining of GOS by Hydrolysis From Vegetal Matrices

Plant based GOS, with α-galactosidic linkages instead of β- ones, are vastly distributed and ubiquitous in the plant kingdom (202). Raffinose, a trisaccharide (Gal-Glc-Fru) is the smallest RFO. Further elongation with Gal residues leads to the DP4 stachyose (Gal-Gal-Glc-Fru), verbascose (DP5), ajugose (DP6), etc. (**Figure 4**) (203). Relevant amounts of α-GOS occur especially in generative parts of plants, such as seeds and fruits; GOS have diverse functions such as physiological protection, germination inhibition under low water availability conditions, and play a role in cold acclimation of many plants (204).

Sugars belonging to the raffinose family have been implicated as protective agents in the cellular dehydration tolerance in plant seeds. Experiments on liposome preservation have demonstrated that the effect of degree of polymerization since RFO were progressively better to stabilize liposomes against leakage of

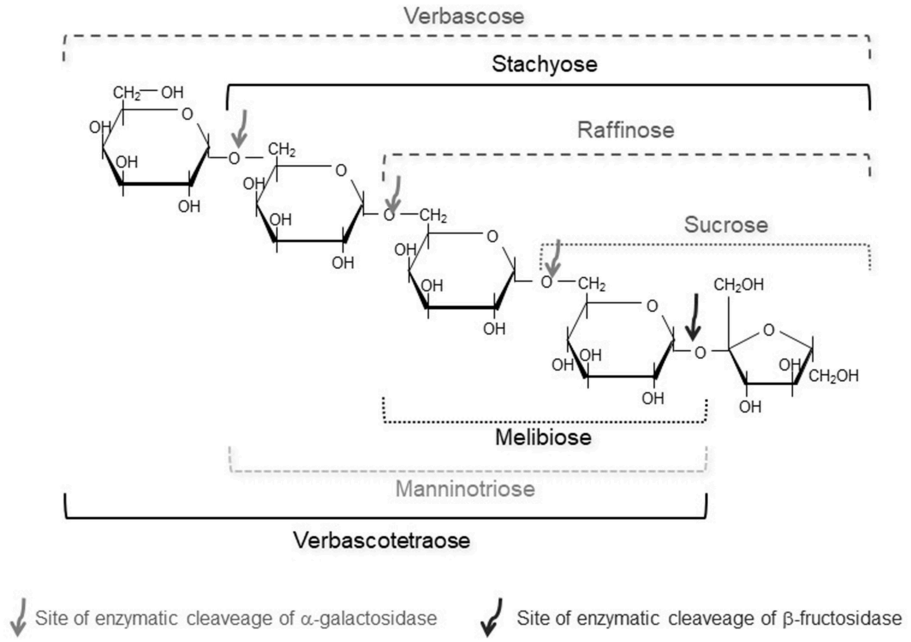

FIGURE 4 | Structures of α-GOS with (Raffinose family, RFO) and without a terminal fructose.

aqueous content and against membrane fusion after rehydration, due to the higher glass transition temperature of the longer chain oligosaccharides (205).

α-GOS can be obtained by extraction from plants, mainly from legume seeds (pulses), such as soybean, lupin, lentil, chickpea, pea and cowpea. α-GOS from soybean are the only legume oligosaccharides in the market and the main producer is Japan. More recently, a French company, Olygose, has developed a type of GOS called Alpha-GOS®. Previously, this compound was a by-product of pea protein production. After research was conducted on the effectiveness of GOS as a prebiotic, Olygose began to produce Alpha-GOS® intentionally, from peas sourced from local farmers in France.

Extractable amounts vary from 1 to 10%, depending on species and cultivar (206, 207). Espinosa-Martos found that GOS content of soybean seeds vary with the degree of maturity. Immature seeds contain less amount of GOS than fully matured ones, but no influence of biological or intensive agricultural practices in GOS content were reported (208).

Unlike FOS, there is no inulin equivalent (no long polymer) from which GOS could be obtained by hydrolysis. DP3 and DP4 are the most abundant GOS but chains of DP7 have been extracted from chickpeas. Usually sucrose is extracted along GOS which some authors claim can be purified by ethanol precipitation (209). However, others found no evidence that sucrose and soy galacto-oligosaccharides could have a differential behavior, both having a similar distribution between the two eluents: water and 70% ethanol. Kim et al. (210) optimized the conditions for oligosaccharide extraction and evaluated an ultrafiltration system for the purification of galacto-oligosaccharides from defatted soybean meal. Their main conclusion was that their system was more efficiency in the removal of protein than in the concentration of oligosaccharides, and no different distribution of GOS and sucrose is observed.

Both extraction and purification procedures must be optimized for each matrix, based on its composition. Considering that seeds are usually rich in lipids, a deffatening step must be performed prior to sugar extraction. Seeds are also high protein parts of the plant and soluble proteins and peptides are normal heavy contaminants on a first extraction. Soluble fiber, such as pectins, are also present on the aqueous extracts (211).

The viability of industrial production of GOS by extraction from natural sources depends strongly on the demands of the application, concerning purity. In order to achieve high purity, a complex set of procedures must be implemented, each step leading to loss of yield.

Like β-GOS, α-GOS are not hydrolyzed in the upper part of the human gastrointestinal tract, due to the absence of the enzyme α-galactosidase. In the colon, they are fermented together with soluble dietary fibers by the colon microbiota, generating significant amounts of short-chain fatty acids (212). These fermentation substrates stimulate the growth of lactobacilli and bifidobacteria and the decrease of enterobacteria in the intestinal microflora. This prebiotic action is beneficial for the host's well-being and health (213, 214). However, fermentation also produces gases (carbon dioxide, hydrogen and methane) that generate bloating and flatulence. Indeed, GOS of RFO are considered an important factor in the development of flatulence caused by consuming legumes (215). On the contrary, melibiose did not promote gas formation, thus suggesting that the fructose moiety present in raffinose was responsible for the gas production (**Figure 4**) (216).

Recently, research has been devoted to so-called "alternative" RFOs in plants. These are novel plant GOS that did not get much attention in the past. The stachyose derivative manninotriose (Gal-α(1→ 6)-Gal-α(1→ 6)-Glc) (**Figure 4**) was found for the first time as main carbohydrate in a garden weed *Lamium purpureum* known as deadnettle (217).

This non-fructosylated raffinose family of α-GOS includes melibiose, manninotriose and verbascotetraose and has been found naturally in foodstuffs (218–220). *In natura*, they may be the result of the activity of plant acid invertases (β-fructosidases), which are able to split sucrose into fructose and glucose by hydrolysis of the 2 → 1 glycosidic bond. This appears to be the base of the recent commercial product of Olygose, mentioned before, that starts with RFO from peas and uses invertases to remove the terminal fructosyl unit from the α-GOS chain.

The European Food Safety Authority (EFSA) analyzed the claim that this group of α-GOS, like RFO and β-GOS, is resistant to hydrolysis and absorption in the small intestine and decided in favor (221); at the same time, they do not have the same gas production negative effects.

OTHER SUBSTRATES FOR THE SYNTHESIS OF FOS AND GOS

Nowadays, Life Cycle Assessments (LCA) and the treatment of by-products from the food industry are gaining importance because of the environmental concern. In this context, using sucrose and/or lactose arising from different by-products or underutilized materials has acquired great importance. As sucrose and lactose are highly available in such kind of products, different attempts have been carried out to use them as raw materials for the synthesis of FOS and GOS, thus adding value to these underutilized products.

Some examples of products available for the synthesis of FOS include carob flour, containing *ca.* 50% sucrose, which has been used as substrate with similar yields than pure sucrose in equivalent concentrations (222). The use of grape must, mainly composed of glucose and fructose, for the synthesis of FOS is a recent and very interesting strategy to add value to a by-product highly available in wine producing countries (223). In addition, sugar syrup and molasses from beet processing containing sucrose were reported to be low-cost and available substrates for the enzymatic synthesis of FOS (54).

In turn, using by-products rich in lactose has been a quite extended strategy for the synthesis of GOS. This is the case of whey permeate. Whey is the by-product remaining from the production of cheese. It is majorly composed of proteins of high biological value (i.e., β-lactoglobulin) and lactose. Whey is generally spray-dried and powders are manufactured as three main products (136): whey protein concentrate, containing 70–85% of the milk whey proteins and 50% of the milk lactose; Whey protein isolate, containing more protein (90–98%) than whey protein concentrates; Whey permeate, essentially composed of lactose and some minerals.

Whey proteins are usually incorporated in the formulation of bakery, meat and dairy products, as well as in infant and sportive food products. The remaining whey permeate is currently used for the production of refined lactose. The obtaining of GOS from whey permeate enables the valorization of whey surplus that economically are not feasible to dry (50). In this regard, milk, sweet and acid whey have also been used as substrate for the synthesis of GOS (149). In addition, different attempts have been used to obtain GOS from whey permeate. Golowczyc et al. (224) used this by-product first to obtain GOS, and then as culture and dehydration medium for probiotic lactobacilli. In turn, Nestle company uses demineralized sweet whey permeate as a food grade source of lactose for the synthesis of GOS. To this aim, the partially demineralized whey permeate containing lactose is evaporated to achieve 50% total dry matter, and then incubated with beta-galactosidases from *A. oryzae* to obtain GOS with DP within 2–5. After synthesis, the enzyme is denatured and inactivated by heating, and the products, containing GOS, mono and disaccharides, purified by membrane nanofiltration, and finally dehydrated.

PROPERTIES AND APPLICATIONS

As mentioned before, the main characteristic of FOS and GOS is their prebiotic effect: both of them are non-digestible food ingredients that selectively stimulate the growth and/or activity of potentially health-enhancing intestinal bacteria (6). Short chain FOS and GOS (DP<5) were especially recognized to encourage the growth of beneficial bacteria in the colon. They act as fermentative substrates, and undergo fermentation in the colon of the host (42, 114). This capacity discourages the growth of potential pathogens in the colon, enhancing the defense mechanisms of the host and protecting against enteric infections. Additionally, this increases mineral absorption and immunomodulation for the prevention of allergies and gut inflammatory conditions; furthermore, they are being investigated as possible reducers of risk factors for colon cancer (4, 42).

Strongly related to the non-digestible characteristic, FOS and GOS are identified as dietary fiber. The European regulation on food labeling obliges the manufacturers to identify these ingredients as dietary fiber (42, 114). In fact, the recent legal definition of fiber is "carbohydrate polymers with 3 or more monomeric units, which are neither digested nor absorbed in the human small intestine obtained from food raw material by physical, enzymatic, or chemical means and which have a beneficial physiological effect demonstrated by generally accepted scientific evidence" (225). Among their nutritional properties, GOS and FOS are carbohydrates that reduce basal hepatic glucose production without any effect on insulin stimulated glucose metabolism, which makes them suitable for diabetic diets (226, 227). Furthermore, they affect lipid metabolism control counteracting triglyceride metabolism disorder and reducing free cholesterol level (42, 227).

Beyond their already known nutritional and prebiotic properties, FOS and GOS have technological properties that are strongly determined by their composition. Both inulin and oligofructose are quite stable toward disadvantageous

technological conditions, namely low pH, high temperatures and low dry solids conditions. In extreme conditions, FOS and inulin are not hydrolyzed when the pH is above 4.5 and the storage temperature is below 10°C. The greater the degree of polymerization, the more stable the oligosaccharide. On this basis, they were used in a wide spectrum of technological applications either as syrups or as powders.

The (2→ 1) glycosidic bonds of inulin make it indigestible to humans and it can therefore be used as a low-calorie sweetener, fat replacer and dietary fiber (228). Short chain FOS are those used for sugar reduction. The technical properties of oligofructose, such as solubility, taste and viscosity, make it a suitable ingredient to reduce the sugar content and increase the fiber content of many food products (i.e., jams, candies, gums, marshmallows) without affecting their organoleptic properties (42, 117, 122). FOS and inulin have been successfully incorporated as sugar replacers in the formulation of dairy products (mainly in yogurts) following the concept "sugar out, fiber in" and "fat out, fiber in." Bakery products, including bread, cookies, cakes and muffins, are other group of products that have benefited from the addition of FOS and inulin in replacement of sugar. Cereals (i.e., breakfast cereals, cereal bars) represent another food category that suits the "sugar out, fiber in" concept, and in which oligofructose has been adequately used in replacement of part of the sugars, leading to products resembling the sugar texture very closely. For example, due to its excellent binding properties and good moisture retention, oligofructose is currently used as a binder of granola bars, leading to an improvement of their shelf-life (oligofructose acts as a humectant, inhibiting the hardening during storage).

Inulin is able to form gels, whose rheological properties are directly related with their crystallization behavior. The primary non-spherical inulin crystallites combine to more or less spherical aggregates which interact to form a weak structured gel where a significant amount of water is immobilized. When inulin is incorporated in a food product the formation of these crystalline aggregates results in an enhanced creaminess and mouthfeel even at dosages much lower than those needed for gel formation (42, 70, 229). These properties make them excellent textures modifiers. Indeed, the addition of inulin to a low fat food product improves his creaminess and texture. The fat replacement and texturizing properties are related to the particle gel behavior. Hence, inulin is an excellent fat-replacer for water containing food systems, where inulin is present as small particles mimicking the mouthfeel and mouthcoating properties of fat. After shearing, inulin particles are formed with a size between 1 and 3 μm which is also the size of fat droplets after homogenization. This property enables the reduction of the caloric content of many products, including dairy products (yogurts, dairy desserts, custards, ice-creams), bakery (cake systems, puff-pastry, croissants, scones). Another category of foods benefiting from the fat-replacement properties of inulin are emulsified meat products, sauces, prepared meals, meal replacers, sausages and pates, which can be obtained with a creamier and juicier mouthfeel and improved stability thanks to the better water immobilization when replacing fat with inulin. Finally, the solubility of inulin and FOS makes them suitable to enrich beverages (dairy beverages, dairy analogs based on soy, rice, almonds or oat, near waters, fruit beverages), converting them in fiber enriched ones.

As a whole, inulin and FOS are natural ingredients highly versatile, whose applications are beyond their functional properties, making them very attractive in the food industry. The combination of the nutritional properties of fiber with the possibility to reduce sugar and fat give fructans a unique position in the ingredient world.

Regarding GOS, Japanese companies were pioneers in introducing them to the market, during the 1990s. At present, most of the applications of GOS are associated to their incorporation into infant products, with the aim of formulating products that more closely approximated human milk. Although their incorporation into food products is clearly regulated in the legislations of USA, European Union, Australia, New Zealand, Argentina and Brazil, their incorporation into other food products is rather limited in comparison to that of FOS. In this regard, in Austria, Finland, Italy, Belgium, the Netherlands and Japan, GOS are used as food ingredients in the formulation of dairy products, fruit juices, bread and bakery products, meal replacers, fermented and flavored milks, and cereal bars. Food for elderly and hospitalized people and poultry, pig and aquaculture products are among other applications of GOS as ingredients (114).

Similar to FOS, the composition of GOS determines their physico-chemical properties as food ingredients. GOS are usually commercialized as mixtures of oligosaccharides (>55%), lactose (<20%), glucose (<20%), and a small amount of galactose, in powder or high concentrated syrups. As GOS have the capacity of remaining stable at high temperature treatments (up to 160°C) and at low pH (2–3) (117), they are considered more stable than FOS (230). The shelf-life of GOS exceeds 18 months without microbial spoilage. GOS containing monosaccharides have relatively low Tg (*ca.* 50°C), thus making them very difficult for spray-drying processes. To counterbalance this disadvantage, the use of whey protein concentrates or maltodextrins has been reported (231). In spite of that, mono and disaccharides present in the matrices make the products highly hydroscopic, so that, they must be stored under dry conditions. This hygroscopic character (that is, humectant properties) makes them suitable ingredients to prevent the excessive drying of bread and other bakery products, thus providing a better taste and texture.

One of the most important applications of GOS is as ingredients for infant formulas. Basically they are added to mimic human milk oligosaccharides, which are claimed to be responsible for a number of physiological effects that impact on the development of newborns (4, 197, 232–234). Additionally, in the food industry, GOS are used as sweeteners, not only in such formulas, but also in fermented products (as milk products and breads), jams, refreshing water and fruit juices (115). Regarding fermented products, GOS are especially suitable for them because of their stability. For example, during bread making GOS resisted yeast fermentation and baking conditions. What is more, the taste and texture of bread remained preserved (117).

In the case of yogurt, GOS besides of being unchanged during the fermentation lactic acid bacteria, studies with consumers suggested that the yogurt with GOS had better sensory attributed (mouthfeel experience) than yogurt without GOS (234). In the case of beverages, particularly fruit juices and soft drinks, GOS are preferred to be incorporated as prebiotic ingredient due to their acid stability and their ability to form clear solutions (213).

Because plant based GOS are not produced from dairy, they are completely lactose free. Growing infant formula demand in China and India as well as application growth in cereals, ice creams and dairy replacement products is expected to have a positive impact on plant based GOS research and development in the near future.

As it was mentioned, besides their application in the food industry, GOS are also relevant in the healthcare industry as constituents in clinical nutrition products (234). These types of products are food and beverages designed for people with a lowered defense system who have specific nutritional needs. These kind of products often contain fiber (both insoluble and soluble) to provide an intestinal function as close as possible to normal food and to prevent constipation or diarrhea. In this sense, from a nutritional point of view, GOS are assumed to be fiber for being non-digestible polysaccharides, so they are suitable for use in different types of medical nutrition concepts, including tube- and sip feed and powdered supplements. Moreover, their stability is extremely important for liquid formulas. In many cases, patients express lactose intolerance. This is why GOS mixtures for this purpose must be lactose-free (213).

GOS prebiotic effect in not limited to human health. They are also interesting ingredients for pet food. They help to maintain animal immune system in right conditions promoting a healthy intestinal environment. Several studies pointed out that GOS consumption favored the generation of lactic acid bacteria such as lactobacilli or bifidobacteria and protected them from pathogens (213). In this line, during the last years, GOS applications in the poultry, pig and aquaculture industries have been rising up. They promote animal's health and growth, improved gut microbial ecology, and reduced diseases, mortality, and fecal odor. Additionally, there was demonstrated that GOS could eliminate methane production by ruminants (114).

CONCLUSIONS

FOS and GOS have been the most investigated compounds with demonstrated prebiotic properties. As they can be obtained either by synthesis or by hydrolysis, they are highly variable in terms of structures. The chirality of GOS obtained by synthesis and by extraction/hydrolysis is opposed (β- in synthesis and α- from extraction/hydrolysis) even though the linkage is identical. Both types of GOS have relevant prebiotic effects. Hence, research work focused on understanding the relationship structure-functionality contributes to the development of the functional food market toward specific health needs.

FOS and GOS are complex structures containing mixtures of oligosaccharides with different degrees of polymerization. Their technological properties strongly depend on their composition which in turn, is a result of the obtainment process. For this reason, an accurate engineering of their production is of great importance to achieve the desired properties. Such engineering depends on many factors, not only technological but also economical. In this regard, the synthesis of FOS and β-GOS has a very important advantage, as substrates (sucrose and lactose) are cost-effective and the reactions can be standardized as there is no variability on the substrates. On the contrary, the natural variability of the raw materials normally used to obtain FOS and α-GOS by hydrolysis can eventually lead to difficulties to standardize the production. However, as α-GOS are assuredly lactose free, their commercial production can be important for relevant market sectors. Standardizing FOS and GOS production by enzymatic synthesis requires the control of the combined effect of reaction conditions (temperature, pH, time, and substrate concentration), enzyme source and activity on the process yield and product composition. In this line, as enzymes are the most expensive input for an economically feasible process, the selected ones are not specific and thus, the reaction conditions must be optimized to achieve a maximum productivity and yield of FOS and GOS. The improvements in immobilization technologies have certainly contributed to overcome this problem in the last years.

Taking into account the advantages and disadvantages of both hydrolysis and synthesis processes, and also the technological properties of the obtained products, an adequate engineering of the processes appears as an important strategy to make the production of FOS and GOS an economically feasible industrial process. This viewpoint is of special interest for small and medium companies, considering the high turnover of FOS and GOS production, which makes the investment in the prebiotic market a very profitable activity.

AUTHOR CONTRIBUTIONS

MU, ET, and AG-Z wrote the issues related to the synthesis of GS and FS, as well as the applications and conclusions. GM and PC wrote the issues related to the obtaining of FS and GS by hydrolysis.

ACKNOWLEDGMENTS

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement N° 777657, and from the Argentinean Agency for the Scientific and Technological Promotion (ANPCyT) (Projects PICT(2014)/0912, PICT start-up (2016)/4808), and PICT(2017)/1344. This research was supported by FCT with funds from the Portuguese Government (Project PEst-OE/QUI/UI0674/2019) through the project M1420-01-0145-FEDER-000005 — CQM+ (Madeira 14-20). AG-Z and ET are members of the research career CONICET. MU is postdoctoral fellow from ANPCyT.

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Conflict of Interest Statement: The 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.

The reviewer SC declared a shared affiliation, with no collaboration, with several of the authors, MU and AG-Z, to the handling editor at time of review.

frontiers in Nutrition

ORIGINAL RESEARCH
published: 05 February 2019
doi: 10.3389/fnut.2019.00002

Extruded Unripe Plantain Flour as an Indigestible Carbohydrate-Rich Ingredient

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There is a growing interest in the development of functional ingredients, including those with high indigestible carbohydrate content. Unripe plantain flour (UPF) is a source of indigestible carbohydrates, type II resistant starch (RS) in particular. A major drawback of UPF, however, is that its RS content decreases sharply after wet heat treatment. Here, we explore the possibility of preparing an extruded UPF-based functional ingredient that retains limited starch digestibility features and high dietary fiber content. Both an unripe plantain pulp flour (UPFP) and a whole (pulp and peel) unripe plantain flour (UPFW) were prepared, extruded under identical conditions and evaluated for their gelatinization degree, total starch (TS), resistant starch (RS), and total dietary fiber (TDF) content; functional properties, such as pasting profile, water retention capacity, and solubility, and oil absorption index were also analyzed. The extruded functional ingredient was added to a yogurt and the rheological characteristics and *in vitro* starch digestibility of the product were evaluated. The extruded UPFW showed a lower gelatinization degree than the extruded UPFP, which may be due to the higher non-starch polysaccharide content of the former. A high TDF content was recorded in both extrudates (12.4% in UPFP and 18.5% in UPFW), including a significant RS fraction. The water retention capacity and solubility indices were higher in the extruded flours, particularly in UPFW, while only marginal differences in oil retention capacity were observed among the products. The addition of UPFP or UPFW (1.5 g TDF, w/v) to a yogurt did not alter the viscosity of the product, an important characteristic for the consumer's approval. Moreover, the composite yogurt showed a relatively low starch digestion rate. Extrusion of UPFs may be an alternative for the production of functional ingredients with important DF contents.

Keywords: extrusion, dietary fiber, starch, carbohydrates, digestibility

OPEN ACCESS

Edited by:
Oswaldo Hernandez-Hernandez, Instituto de Investigación en Ciencias de la Alimentación (CIAL), Spain

Reviewed by:
Juliana Gamboa Santos, Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), Argentina
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Specialty section:
This article was submitted to Nutrition and Food Science Technology, a section of the journal Frontiers in Nutrition

Received: *07 November 2018*
Accepted: *10 January 2019*
Published: *05 February 2019*

Citation:
Garcia-Valle DE, Bello-Perez LA, Flores-Silva PC, Agama-Acevedo E and Tovar J (2019) Extruded Unripe Plantain Flour as an Indigestible Carbohydrate-Rich Ingredient. Front. Nutr. 6:2. doi: 10.3389/fnut.2019.00002

INTRODUCTION

In the last years, the end-use of plantain and other varieties of banana has increased around the world. Only in the present year, SCOPUS database shows 14 publications on the use of banana flour in foods, composite films, and modifications to improve its functional and physicochemical features. The use of plantain flour aims to diversify the consumption of the fruit, minimizing the large quantities currently lost during conveying, maturation, and commercialization. The use of plantain fruit in the unripe state is an interesting alternative, due to its high indigestible

carbohydrate (components of dietary fiber) content, where resistant starch (RS) is the main component (1). There are diverse studies of the use of unripe plantain flour (UPF) in its native state; however, when this flour is incorporated in heat-treated foods, such as bakery products, snacks, and pasta (2–4), the cooking process largely decreases the indigestible carbohydrate content due to RS conversion to digestible starch. However, we have recently shown that the resistant-to-digestible starch conversion degree depends on the food type. For instance, foods with low moisture content, e.g., UPF-containing cookies, exhibit low conversion rate thus retaining a high dietary fiber content (5). Nevertheless, the drawback of the use of UPF in cooked foods can be overcome with the modification of the flour. Hydrothermal treatments, such as heat-moisture treatment and annealing, were used to modify the starch digestibility in UPF. Rodriguez-Damian et al. (6) reported that heat-moisture treatment of UPF increased its RS content to 11% after cooking. Similarly, the RS content in cooked UPF reached 17% after annealing treatment (7). Additionally, esterification of UPF with citric acid preserved its RS content (93.7%) after cooking (8). The above-mentioned studies have focused on the modification of UPF prepared from the pulp of the fruit; however, in order to decrease the production costs and to increase both the yield and dietary fiber content of UPF, the use the whole fruit (pulp and peel) has been suggested. Such an alternative was tested to produce gluten-free spaghetti. Still, no differences were found in the dietary fiber content ($\approx$ 30) of spaghettis elaborated with pulp-derived UPF compared to those made of whole fruit UPF (9).

Extrusion has been used to prepare pre-gelatinized starches that can be solubilized in water at room temperature. The increased solubility and overall digestibility of the pre-gelatinized starch are attributed to the disorganization of starch components in the granular structure, a process that can be enhanced as the gelatinization degree increases in the extruded starch. However, we hypothesized that if UPF is extruded under specific conditions, it can retain high RS content adding to non-starch polysaccharides in the fiber fraction, and may be thus included in foods where further cooking is not necessary, such as yogurt, smoothies, etc. Such a procedure would be in line with the current interest in the development of foods and ingredients with high dietary fiber contents, which derives from the evidences linking diverse health problems, e.g., overweight, obesity, diabetes, cardiovascular diseases, colon cancer, etc., to the generally low consumption of dietary fiber in occidental populations (10).

The aim of this study was to produce a ready-to-use fiber-rich ingredient by extrusion of UPF, and to perform its chemical, functional and physicochemical characterization. The ingredient was also included in a yogurt to evaluate its impact on the rheological and sensory features of the final product.

MATERIALS AND METHODS

Unripe Plantain Flour (UPF)

Unripe plantain fruits were harvested in Tuxtepe, Oaxaca, México. The flours from the pulp and from the pulp and peel were prepared as described by Ovando-Martinez et al. (1) and Patiño-Rodriguez et al. (9).

Extrusion

UPF from pulp (UPFP) and the whole fruit (pulp + peel) (UPFW) were extruded in a single screw-extruder (Beutelspacher, México, City, México) at a constant rate of 75 rpm. The temperature in the three zones of the extruder (first zone of the barrel, blend zone, and end zone) was kept constant at 50°C, conditions that in preliminary studies resulted in adequate rheological properties without promoting complete gelatinization of starch in plantain flours (unpublished data). Two batches were prepared for each type of flour.

Differential Scanning Calorimetry (DSC)

Thermal analysis of gelatinization (in excess water) was assessed to determine the temperatures and enthalpy associated to this phase transition. A 2.0 mg sample was mixed with 7 μl of deionized water. The wetted sample was equilibrated for 12 h at room temperature and subjected to the heating program over a temperature range from 20 to 100°C at a heating rate of 10°C/min in a DSC model 2010 (TA Instruments, New Castle, NJ). The temperatures of the phase transition and enthalpy (area under the phase transition curve) were calculated with the software of the equipment.

Total Dietary Fiber

The total dietary fiber (TDF) content of the raw and extruded samples was determined by the enzymatic method proposed by McCleary et al. (11), which yields DF values including RS, using experimental conditions that resemble the physiological situation. Total dietary fiber (TDF) was also assessed according to AACC method 32-05 (12).

Total and Resistant Starch

Total starch (TS) was measured according to AACC method 76.13 (12) using a total starch kit from Megazyme (Wicklow, Ireland). Resistant starch (RS) was determined following the AACC method 32–40 (12) with the RS kit (Megazyme, Wicklow, Ireland).

Pasting Profile

The pasting profile was evaluated during the cooking of the flours in a stress rheometer (Ar-1500ex, TA Instruments, Dallas, TX, USA) using a starch pasting cell (SPC) with a vanned rotor at 500 s^{-1}. The temperature profile started with a heating ramp temperature of 5°C/min from 50 to 95°C, holding at 95°C for 10 min, cooling ramp temperature of 5°C/min from 95 to 60°C and finally holding at 60°C for 10 min. The starch concentration in the sample was 8% (dry basis).

Water Retention Capacity and Solubility

Water retention capacity was determined according to the method of Hallgren (13). Briefly, 5 ml of water were added to 0.25 g UPF samples (raw and extruded) in pre-weighed centrifuge tubes at room temperature and heated at different temperatures (40–80°C) for 15 min, with shaking at 5 and 10 min. The tubes were then centrifuged for 15 min at 1,000 × g, 10 min. The supernatant was decanted, and the tubes were allowed to drain for 10 min at a 45° angle. The tubes were then weighed and the

gain in weight was used to calculate percent gain as the water retention capacity. Experiments were performed in duplicate.

Oil Absorption Index

The method described by Lin et al. (14) was used to determine oil absorption capacity (OAC). UPF (100.0 ± 0.2 mg) was mixed with 1.0 mL of vegetable oil. The mixture was stirred for 1 min with a wire rod to disperse the sample in the oil. After a period of 30 min in the vortex mixer, tubes were centrifuged at 3000 × g and 4°C for 10 min. The supernatant was carefully removed with a pipette and the tubes were inverted for 25 min to drain the oil and the residue was then weighed. The oil absorption capacity was expressed as grams of oil bound per gram of sample on dry basis. Three replicates were performed for each sample. OAC was calculated by equation:

$$OAC(g/g) = Wr/Wi$$

Where:

$$Wr = \text{residue weight}$$
$$Wi = \text{sample weight}$$

Application of the Functional Ingredient in a Non-heat Processed Product. Viscosity Measurement in Yogurt

For viscosity measurements, an in-house made yogurt was mixed with plantain flours to yield total fiber concentrations ranging from 0.1 to 1.5% (w/v). Steady shear rate sweep was carried out at 25°C in a stress-controlled rheometer AR1500ex (TA Instruments, New Castle, USA), using the standard concentric cylinders fixture (HA AL CONICAL DIN, inside radius = 13.98 mm, outside radius = 15.19 mm, length = 42.05 mm, GAP = 5,920 μm). An ascendant sweep from 0.1 to 100 s^{-1} was chained.

In vitro Starch Digestion

The *in vitro* digestibility was assessed according to the methodology described by Zheng et al. (15). In summary, a sample equivalent to 100 mg dry starch was weighed in a beaker and artificial saliva containing porcine α-amylase (250 U per mL of carbonate buffer, pH 7) and pepsin (1 mg per mL in 0.02 M HCl, pH 2) was added. The mixture was incubated at 37°C for 30 min, followed by a second digestion step performed with a mixture of pancreatin (2 mg per mL) and amyloglucosidase (28 U per mL), at pH 6.0. Samples were taken at different time intervals (5, 10, 15, 20, 30, 40, 50, 60, 90, 120, 240, and 360 min) and mixed with 300 μL of stop solution (0.3 M Na_2CO_3) to prevent further amylase activity in the aliquot. After centrifugation (2,000 g for 5 min), the glucose concentration in the supernatant was determined using a D-Glucose Assay Kit (GOPOD Format from Megazyme, International, Bray, Ireland) according to the supplier instruction. Results are presented as starch hydrolyzed (grams per 100 g dry starch).

Sensory Evaluation

One hundred untrained judges evaluated the acceptability of the yogurt. Panelists were asked to assess their degree of liking using a 9-point hedonic scale, where 9 = like extremely and 1 = dislike extremely. The sensory evaluation was targeted to flavor, texture, odor and general acceptance of the yogurt, using the questionnaire proposed by Agama-Acevedo et al. (16).

Statistical Analysis

Results are presented as mean ± SD (standard deviation). Differences among the means obtained in each of the determinations were evaluated by one-way analysis of variance (ANOVA) with a significance level of $\alpha = 0.05$, followed by Tukey's test using the statistical package Origin Pro 2016 (OriginLab Corporation, MA, USA). Student's *t*-test was used for the statistical analysis of gelatinization degree.

RESULTS AND DISCUSSION

Degree of Gelatinization

The degree of gelatinization was determined in both extruded samples, the unripe plantain flour of the pulp (UPFP) and that from whole fruits (UPFW). The enthalpy and degree of gelatinization values are shown in **Table 1** and the DSC-traces in **Figure 1**. Only minor differences were observed in the average gelatinization temperatures (Tp) of both flours and extrudates (**Table 1**), although the extruded UPFP showed a slightly lower value than its UPFW counterpart. This pattern is associated with the higher total starch content in the former sample (**Table 2**) which is also reflected in the small difference registered in the degree of gelatinization. No information is available in the literature on the degree of gelatinization of extruded UPFs. Usually, extruded flours of different sources show a complete gelatinization, but the conditions used in the current extrusion protocol seem to partially maintain the structural arrangement of starch components of the granules. The difference between both samples can be explained by the dietary fiber content (non-starch polysaccharides) in the flour containing fruit peel, which may have restricted the heat flow transmission during extrusion, as reported in spaghettis elaborated with green banana flour and non-starch polysaccharides (hydrocolloids), resulting in a dense packing and physical entrapment of starch in networks that restrict both the swelling of granules and their accessibility to digestive enzymes (15).

TABLE 1 | Temperature (Tp) and enthalpy of gelatinization (ΔH), and gelatinization degree determined by differential scanning calorimetry of native and extruded flours of unripe plantain pulp (UPFP) and whole fruit (UPFW).

Sample	Tp (°C)	ΔH (J/g)	Gelatinization degree (%)
UPFW	79.6 ± 0.2^a	8.4 ± 0.2^a	–
UPFP	78.7 ± 0.2^a	7.6 ± 0.2^a	–
Extruded UPFW	85.2 ± 0.2^b	3.9 ± 0.7^b	63^a
Extruded UPFP	83.9 ± 1^b	2.4 ± 0.2^b	68^b

Values represent mean ± SD of four determinations. Mean values in a column followed by different letters are significantly different ($P < 0.05$) compared by Student's t-test.

Starch and Dietary Fiber Content

Total starch (TS) content was higher in the flour of the pulp (UPFP) than in the peel-containing preparation (UPFW), and the same pattern was observed in the extruded samples (**Table 2**). Extrusion resulted in significantly lower TS contents, which may be related to depolymerization of some amylose chains and formation of transglucosidation products (17), a process that renders alpha-glucans unavailable to amylolytic enzymes (18). The RS in the raw flours was high (around 55% of the TS content) but decreased after extrusion. This reduction in RS content is explained by the above-discussed partial gelatinization of plantain starch, a change that runs in parallel with increased susceptibility to amylolysis (10, 17). However, the RS content remaining in the extruded ingredient obtained from both UPFs (4.8–5.8%) can still be considered high compared with the dietary fiber-associated starch in conventional food items, such as whole wheat bread (1.9–2.8%) and cooked legumes (3–7%) (10, 19).

The total dietary fiber (TDF) content of the flours, determined with a method for products "as eaten" (11), decreased from 49 to 57% in the native preparation to 12–18.5% in the extruded flours (**Table 2**). In spite of this marked effect of the extrusion treatment, the remaining DF contents were still important. These results may be attributed to the protective effect that non-starch polysaccharides exert against complete gelatinization of starch granules, which allows for a significant retention of the RS component of DF. The DF assessed with the AACC method (12), which measures non-starch polysaccharides and only a minor portion of indigestible starch (i.e., type 3 RS), confirmed that DF in the extruded UPFW is mainly composed of non-starch polysaccharides (11.6%) (**Table 2**), while the total RS retained (5.8%) fills the gap to the overall 18.5% DF content of the product.

The high DF content recorded in both extruded samples, particularly UPFW, suggests the products' potential as functional ingredients that can be added to a variety of foods, like salads, fruits, smoothies, yogurt, and breakfast cereals. The possible use of the extrudates in other types of food such as salad dressings, emulsions, bakery products and snacks should be further explored.

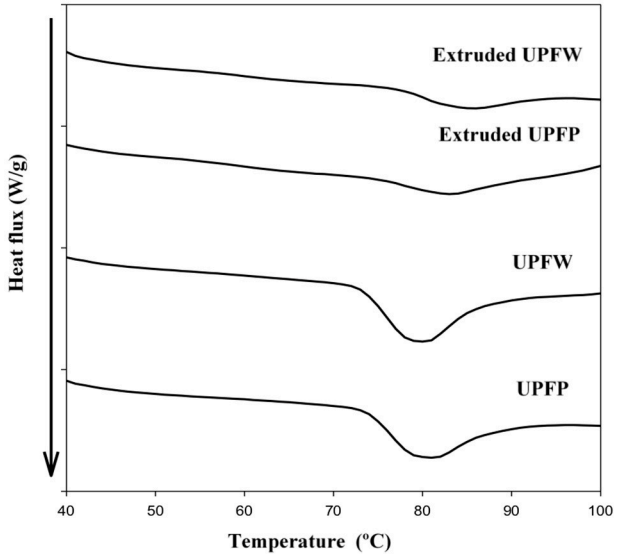

FIGURE 1 | Differential scanning calorimetry of native and extruded flours of unripe plantain pulp (UPFP) and whole fruit (UPFW).

TABLE 2 | Total starch (TS), resistant starch (RS), and dietary fiber (DF) content in native and extruded flours of unripe plantain pulp (UPFP) and whole fruit (UPFW) (g/100 g).

Sample	TS	RS	DF[1]	DF[2]
UPFW	74.5 ± 1.1[a]	46.6 ± 0.5[a]	57.2 ± 0.9[a]	12 ± 0.6[a]
UPFP	85.4 ± 0.9[b]	42.8 ± 0.6[b]	49.6 ± 1.0[b]	7 ± 0.5[b]
Extruded UPFW	70.8 ± 0.7[c]	5.8 ± 0.6[c]	18.5 ± 0.6[c]	11.6 ± 0.7[a]
Extruded UPFP	82.5 ± 0.8[d]	4.8 ± 0.7[d]	12.4 ± 0.7[d]	6.8 ± 0.5[b]

DF[1] = Method of (11); DF[2] = Method of 32-05 (12).
Values are reported as mean ± SD of four determinations. Mean values in a column followed by different superscripts are significantly different (P < 0.05).

Pasting Profile

The pasting profile of both raw flours resembles those exhibited by pure starches (**Figure 2**), which agrees with the fact that starch is the main component of the flours. The UPFW presented the largest viscosity peak, indicating that the starch granules undergo extensive swelling before they brake, possibly due to the protective effect of the non-starch polysaccharide components of the preparations. The breakdown and setback in both raw flours were similar, indicating that their starch granules broke and reorganized in a similar pattern. UPFW showed the highest final viscosity, which is related to the network produced during cooling, a process that also involves cognate non-starch polysaccharides. The extruded samples developed notably lower viscosity, with no defined viscosity peak, a

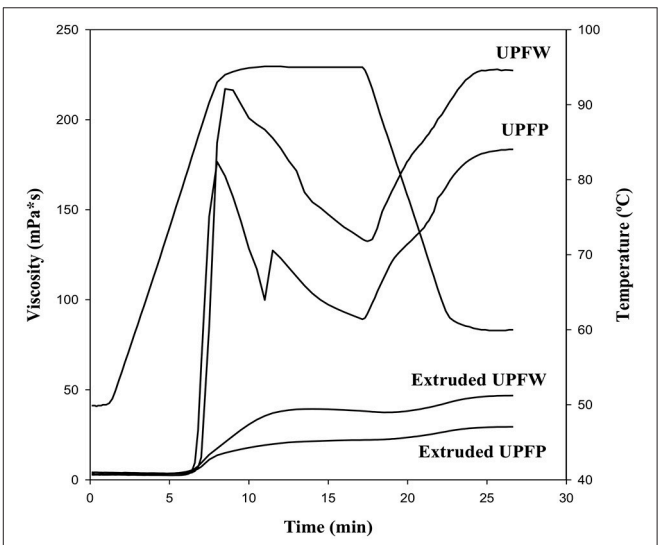

FIGURE 2 | Pasting profile of native and extruded flours of unripe plantain pulp (UPFP) and whole fruit (UPFW).

behavior that is in accordance with the previous heath treatment they underwent. Extruded UPFW exhibited higher maximum viscosity than extruded UPFP. The larger viscosity increase observed for the UPFW extrudate is probably due to the higher concentration and different physicochemical features of the non-starch polysaccharides present in this sample.

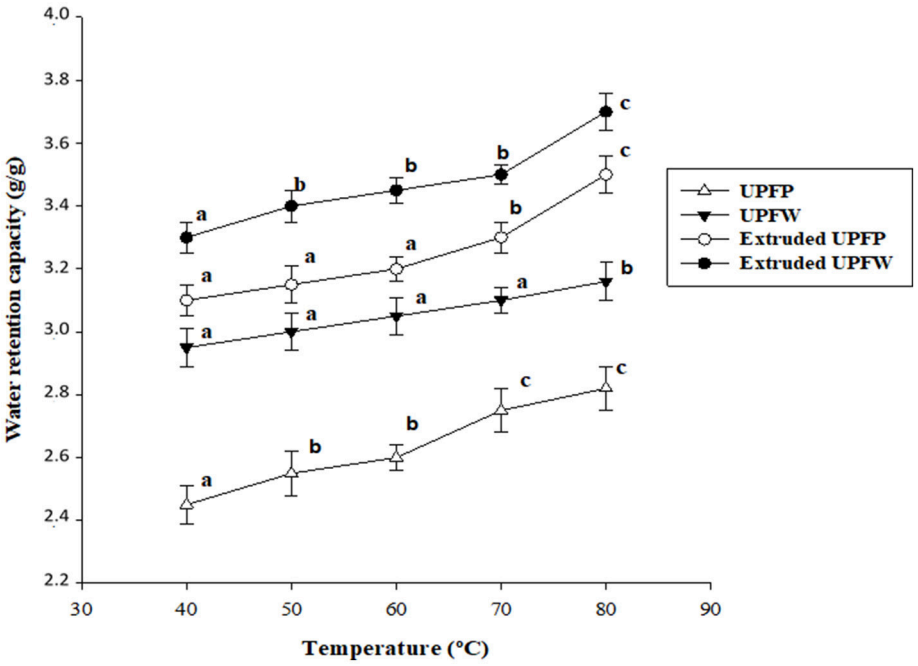

FIGURE 3 | Water retention capacity at different temperatures of native and extruded flours of unripe plantain pulp (UPFP) and whole fruit (UPFW). Different superscript letters on the same curve indicate statistically significant differences ($P < 0.05$).

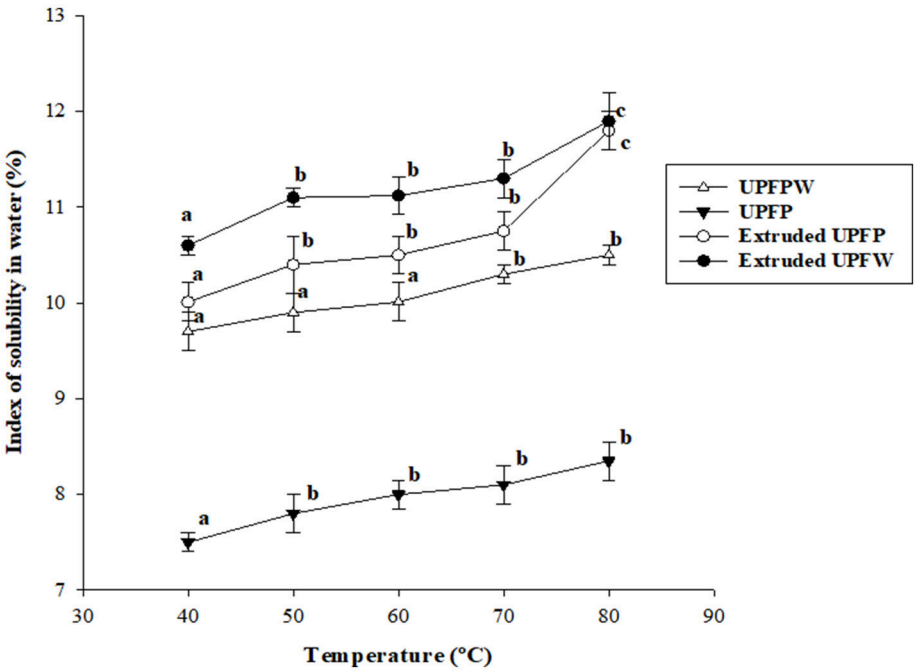

FIGURE 4 | Solubility at different temperatures of native and extruded flours of unripe plantain pulp (UPFP) and whole fruit (UPFW). Different superscript letters on the same curve indicate statistically significant differences ($P < 0.05$).

Water Retention Capacity and Solubility

Both raw flours showed similar water retention capacity (WRC) patterns, i.e., increased WRC concomitant with temperature increments (**Figure 3**). The higher WRC recorded for UPFW is related to its higher content of hydrophilic components, i.e., starch and dietary fiber (**Table 2**). The extruded UPFW sample behaved similarly to its raw counterpart, but always with higher WRC values at the different temperatures. Also, the WRC values were higher for the extruded UPFW than for extruded UPFP. Again, the difference between the two extrudates can be related to the higher total starch and DF content of the UPFW extrudate (**Table 2**). Additionally, as discussed above, UPFW contains a fraction of un-gelatinized starch that may be affected by the additional heat treatment involved in the WRC test, leading to further swelling of those starch granules with the concomitant increase in the WRC. This result reinforces the idea that extruded UPFs may be suitable as RS/DF ingredients in products that do not require further heat treatment before consumption.

The temperature-dependent solubility changes of all samples (**Figure 4**) followed a trend that resembles that of WRC. The extruded UPFP was lower than extruded UPFW, although similar values were determined at the highest temperature tested. The difference between extrudates is related to the higher starch content and degree of gelatinization in UPFW, as starch components (mainly amylose) are solubilized during heating.

Oil Absorption Index (OAI)

The raw flours showed similar OAI (**Table 3**). The extruded samples presented a slight increase in the OAI compared to the native ones, and no difference was found between both samples. The higher values observed for the extrudates can be due to the disorganization of starch components during extrusion which results in partial release of amylose chains prone to form inclusion complexes with lipids. The OAIs shows that the starch-rich extruded plantain flours have potential as a fat replacer, given their higher OAI compared to extruded maize flour used to stabilize emulsions (20) and extruded wheat flour used as fat replacer in batter (21). Modified starches have been used in products of reduced-fat content (22), and recently an extruded pre-gelatinized flour was proposed as fat replacer in mayonnaise (20).

TABLE 3 | Oil absorption index of native and extruded flours of unripe plantain pulp (UPFP) and whole fruit (UPFW).

Sample	Oil absorption index (g oil/ g solid)
UPFW	2.55 ± 0.4^{a}
UPFP	2.53 ± 0.5^{a}
Extruded UPFW	2.68 ± 0.5^{b}
Extruded UPFP	2.65 ± 0.7^{b}

Values are reported as mean ± SD of four determinations. Mean values followed by different letters are significantly different ($p < 0.05$).

Rheological Characteristics of Yogurt

The effect of the addition of the extruded flours on the viscosity of a yogurt is shown in **Figures 5**, **6**. The viscosity of all samples decreased with the increase in the shear rate, indicating a pseudoplastic behavior. The addition of the extrudates produced a concentration-related increase in the viscosity, without altering the pseudoplastic feature. The impact of the extrudates on viscosity can be attributed to the starch and non-starch

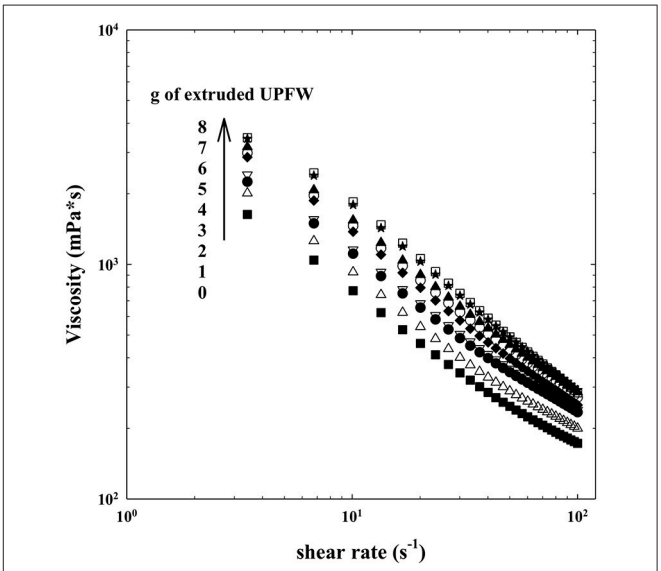

FIGURE 5 | Viscosity of yogurt added with extruded flour of whole unripe plantain (UPFW).

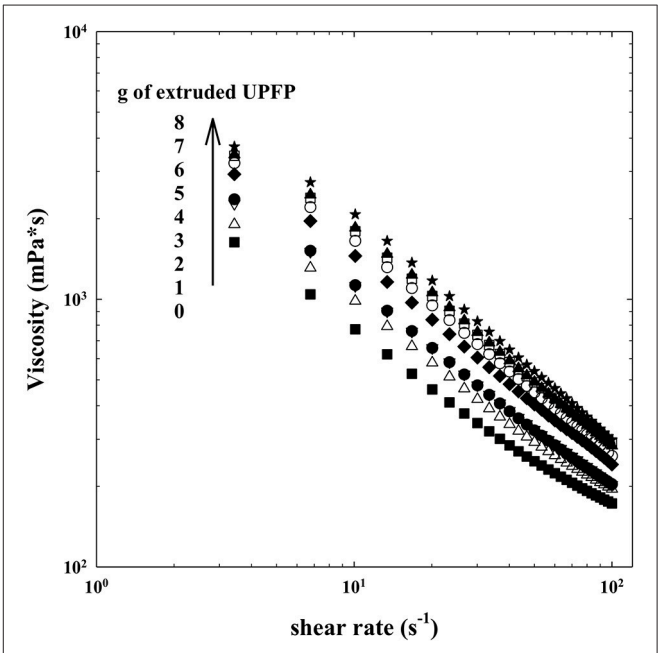

FIGURE 6 | Viscosity of yogurt added with extruded flour of unripe plantain pulp (UPFP).

polysaccharides present in both samples. The effect on viscosity was higher for the UPFW extrudate. It can be suggested that the fiber components in the whole fruit (e.g., cellulose, hemicellulose) are responsible for the higher viscosity values due to their network-generating ability in this food matrix (23). A similar pattern was found in soy yogurt added with insoluble fiber (24). Power law model was used to fit the experimental data with a $R^2 > 0.99$ (**Table 4**). The addition of the extrudates at equivalent concentrations did not change the n value, but the consistency coefficient (k) was higher in the yogurt with extruded UPFW, which corroborated the larger viscosity increase this extruded preparation. It interesting to note that here-reported results on the effects of adding unripe plantain-derived flours to yogurts show an inverse pattern when compared to those reported for a yogurt added with resistant starch or β-glucans, where the n value decreased as the polysaccharide concentration increased (25). Evidently, the chemical structure and physicochemical characteristics of the fiber ingredient chosen are important determinants of the sensory-related characteristics of fiber-enriched products.

In vitro Starch Digestion Rate

The *in vitro* starch hydrolysis curves for yogurt added with extruded UPFW and UPFP are shown in **Figure 7**. Both yogurt preparations showed similar hydrolysis curves with a relatively rapid starch digestion, although it was slower than for the gelatinized starch reference. There may be several reasons for the reduced rate of digestion of the plantain-derived flours. Non-starch polysaccharides present in the food matrix (extruded) can hamper the enzyme (amylase) diffusion and limit the swelling of the substrate (starch granule) resulting in only partial gelatinization; both phenomena retard starch hydrolysis. Also, it has been reported that cellulose and other fibers may inhibit α-amylase activity, attenuating starch hydrolysis (26, 27). Moreover, the extrusion of plantain flours produces partial amylose lixiviation from the granules, and "ghost" granules can produce physical entanglement (low-order starch matrices) that may modulate the hydrolysis by digestive enzymes (28). Since moderate starch digestion rates are considered beneficial in terms of the postprandial metabolic responses to foods (10, 17), the addition of UPFW and UPFP to yogurt may be a positive feature besides increased DF content.

TABLE 4 | Rheological properties of yogurt added with extruded flours of unripe plantain pulp (UPFP) and whole fruit (UPFW).

g of extruded UPFP/60 mL yogurt	*K* (mPa*s-1)	*n*	R^2
0 (Control)	3881.50 ± 0.02^a	0.30 ± 0.0^a	0.9974
1	4663.37 ± 0.02^b	0.29 ± 0.0^a	0.9973
2	5134.52 ± 0.02^c	0.31 ± 0.0^a	0.9987
3	5582.13 ± 0.02^d	0.30 ± 0.0^a	0.9990
4	6684.97 ± 0.01^e	0.28 ± 0.0^a	0.9988
5	6900.80 ± 0.01^f	0.29 ± 0.0^a	0.9988
6	7301.29 ± 0.01^g	0.29 ± 0.0^a	0.9989
7	8077.92 ± 0.01^h	0.26 ± 0.0^a	0.9971
8	8910.45 ± 0.01^i	0.26 ± 0.0^a	0.9958
g of extruded UPFW/60 mL yogurt			
0 (Control)	3881.50 ± 0.02^a	0.30 ± 0.0^a	0.9974
1	5075.75 ± 0.01^b	0.27 ± 0.0^a	0.9985
2	5697.70 ± 0.01^c	0.27 ± 0.0^a	0.9996
3	5975.85 ± 0.01^d	0.26 ± 0.0^a	0.9994
4	7561.22 ± 0.01^e	0.25 ± 0.0^a	0.9990
5	8077.92 ± 0.01^f	0.26 ± 0.0^a	0.9971
6	8910.45 ± 0.01^g	0.26 ± 0.0^a	0.9958
7	9067.75 ± 0.01^h	0.25 ± 0.0^a	0.9984
8	9761.12 ± 0.01^i	0.26 ± 0.0^a	0.9938

K, consistency index; n, flow behavior index and R^2, correlation coefficient. Values with different superscripts within the same column are significantly different ($P < 0.05$).

Sensory Evaluation

The results of the sensory evaluation study (**Table 5**) showed that control yogurt (no flour added) received the highest score, whilst the yogurt with UPFW had the lowest acceptability, although it was not too different from that of UPFP-containing yogurt. The presence of fruit peel in the extruded flour can be a reason

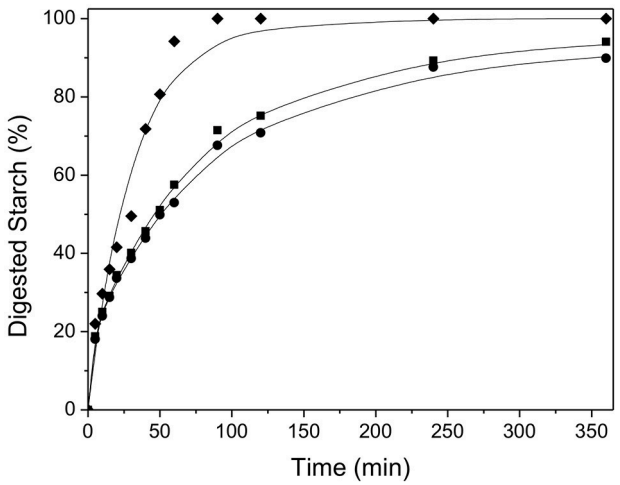

FIGURE 7 | Hydrolysis kinetics and fitting curve for yogurt with the extruded flours of whole unripe plantain (UPFW, •) and flours from the fruit pulp (UPFP, ■). Regular maize starch (gelatinized by 10 min boiling) was used as reference (♦). The lines depict least-squares numerical fitting. Symbols (■, •, ♦) are experimental data.

TABLE 5 | Sensory evaluation of yogurt added with extruded flours of unripe plantain pulp (UPFP) and whole fruit (UPFW).

Yogurt sample	Overall acceptability*
Extruded UPFP	4.7 ± 0.26^a
Extruded UPFW	3.6 ± 0.21^b
Control	± 0.32^c

**Mean ± SD, n = 100. Means in column not sharing the same letter are significantly different at $p < 0.05$. Control sample is natural yogurt (no UPF added).*

for the low acceptability, as it is well-known that addition of any ingredient in products like yogurts, smoothies, fruits and salads, modify the sensory characteristics and acceptability of the product; however, it should be noted that the appreciation for these products improves after regular consumption and/or addition of flavor (16). On the other hand, the high dietary fiber content in the yogurt, which portraits it as a "healthy food," can be attractive for consumers with more flexible acceptability criteria. Nevertheless, the sensory features of the yogurt added with the UPFW may be improved, for instance with addition of flavor, an aspect to be explored in future studies.

CONCLUSIONS

Extrusion of flours from unripe plantain, using either the whole fruit (UPFW) or the pulp (UPFP), produced ingredients rich in indigestible carbohydrates. The extruded UPFW exhibits higher fiber content (including resistant starch), water retention capacity and solubility than the extruded UPFP. The oil absorption index was relatively high in both extrudates. The addition of the extruded flours to a yogurt resulted in slightly increased viscosity, moderate starch hydrolysis rate and somewhat reduced acceptability of the product. Extruded UPFs represent a potential ingredient with nutritional-functional properties that may be used in ready-to-eat processed foods.

AUTHOR CONTRIBUTIONS

LB-P and DG-V conceived the study. DG-V, LB-P, PF-S, and EA-A performed the experiments and participated in the acquisition of the data. DG-V, LB-P, PF-S, EA-A, and JT carried out the analysis and interpretation of the data. DG-V, LB-P, and JT drafted the manuscript. All authors read and approved the final version of the paper.

ACKNOWLEDGMENTS

We appreciate the economic support from Instituto Politécnico Nacional (IPN), SIP-IPN, EDI-IPN CONACYT-México, and COFAA-IPN. One of the authors (DG-V) acknowledges the scholarship from CONACYT-México.

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Conflict of Interest Statement: The 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.

frontiers in Nutrition

ORIGINAL RESEARCH
published: 22 March 2019
doi: 10.3389/fnut.2019.00030

Effect of α-Glucosylation on the Stability, Antioxidant Properties, Toxicity, and Neuroprotective Activity of (–)-Epigallocatechin Gallate

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(–)-Epigallocatechin gallate (EGCG), the predominant catechin (≥50%) in green tea (*Camellia sinensis*), displays several bioactive properties but its stability and bioavailability are low. In this work, the properties of two α-glucosyl derivatives of EGCG (3′- and 7-O-α-D-glucopyranoside), obtained by enzymatic synthesis, were assessed. The α-glucosylation enhanced the pH and thermal stability of EGCG. The analysis of scavenging activity toward $ABTS^{\cdot}+$ radicals showed that the α-glucosylation at C-7 of A-ring caused a higher loss of antioxidant activity compared with the sugar conjugation at C-3′ of B-ring. The 3′-glucoside also showed higher potential to alleviate intracellular reactive oxygen species (ROS) levels and to boost REDOX activity. The toxicity of EGCG and its monoglucosides was tested in human SH-S5Y5 neurons, RAW 264.7 macrophages, MRC5 fibroblasts, and HT-29 colon cancer cells. Interestingly, the 3′-O-α-D-glucoside increased the viability of neural cells *in vitro* (2.75-fold at 100 μM) in the presence of H_2O_2, whilst EGCG gave rise only to a 1.7-fold enhancement. In conclusion, the α-glucoside of EGCG at C-3′ has a great potential for nutraceutical, cosmetic and biomedical applications.

Keywords: glycosylation, tea polyphenols, antioxidants, catechins, neuroprotective properties

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Nutrition and Food Science Technology, a section of the journal Frontiers in Nutrition

Received: *13 November 2018*
Accepted: *01 March 2019*
Published: *22 March 2019*

Citation:
Gonzalez-Alfonso JL, Peñalver P, Ballesteros AO, Morales JC and Plou FJ (2019) Effect of α-Glucosylation on the Stability, Antioxidant Properties, Toxicity, and Neuroprotective Activity of (–)-Epigallocatechin Gallate. Front. Nutr. 6:30. doi: 10.3389/fnut.2019.00030

INTRODUCTION

Plant polyphenols are gaining relevance due to their capacity to delay the appearance of certain degenerative diseases and pathological processes such as Alzheimer's and Parkinson's diseases, schizophrenia, cancer, chronic inflammatory disease, atherosclerosis or myocardial infarction (1–3). Their action is based on the enhancement of the antioxidant system due to their ability to reduce the level of reactive oxygen species (ROS) (4). Many polyphenols are lipophilic scaffolds with rapidly conjugated phenolic OHs that exhibit poor absorption *in vivo*, giving rise to a very low concentration in the circulatory streams (5).

Several polyphenols appear glycosylated in nature (4, 6) and the sugar moiety seems to play a major role in their solubility (7), partition coefficient (8), protection from oxygen, pH, temperature and/or light (9), absorption (10, 11), bioavailability (12), and bioactivity (13). Several studies demonstrated that glycosylation facilitates the diffusion of polyphenols into intestinal enterocytes (12, 14). Other investigations have shown that deconjugation of the glycosyl moiety of glycosylated

flavonoids favors cellular uptake by enterocytes (15, 16). Despite this controversy in the role of glycosylation on bioavailability, there is some consensus that glycosylation increases the stability of polyphenols during gastrointestinal transit after ingestion (17) and also during storage (18). In fact, glycosylation is being exploited as a tool to improve the properties of polyphenols (7, 19–22). Enzymatic synthesis is gaining importance due to its selectivity and the environmentally friendly reaction conditions (23–25).

(–)-Epigallocatechin gallate (EGCG) is the predominant catechin (≥50%) in green tea (*Camellia sinensis*). It possesses antioxidant (26), antihypertensive (27), antitumoral (28, 29), bactericidal (30), and anti-inflammatory (31) bioactivity, among others. However, EGCG undergoes rapid degradation in aqueous solutions (32) resulting in a low bioavailability (33). The two main processes involved in the instability of EGCG are epimerization and oxidative coupling (34). In order to increase its stability and bioavailability (35), and to reduce its astringency for food applications (36), the glycosylation of EGCG has been explored by several groups, mostly by the use of enzymatic catalysis (37–39). Recently, our group reported the enzymatic synthesis of various α-glucosyl derivatives of EGCG by a transglycosylation reaction catalyzed by a cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) (40). Two main α-D-glucosides of EGCG were isolated and chemically characterized: EGCG 3′-O-α-D-glucopyranoside (***1***) and EGCG 7-O-α-D-glucopyranoside (***2***).

In the present work, we have analyzed the effect of α-glucosylation on several properties of EGCG, in particular the pH and thermal stability, the antioxidant and REDOX activities, the toxicity toward several cell lines and the neuroprotective activity. Consequently, the influence of the position of glycosylation on such properties was assessed.

MATERIALS AND METHODS

Enzyme and Reagents

(-)-Epigallocatequin gallate (EGCG) was acquired from Zhejiang Yixin Pharmaceutical Co. (Zhejiang, China). Toruzyme 3.0L, a commercial preparation of cyclodextrin glucanotransferase (CGTase) from *Thermoanaerobacter* sp., was kindly provided by Novozymes. Partially hydrolyzed starch from potato (Passelli SA2) was from Avebe (Foxhol, The Netherlands). ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] and (R)-Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma Aldrich. All other reagents and solvents were of the highest available purity and used as purchased.

Stability Assays

EGCG and its glucosylated derivatives were dissolved at 4 mg/mL in 20 mM sodium phosphate buffer (pH 6.7) and incubated at 60°C. At intervals, aliquots of 150 μL were withdrawn, diluted 2-fold with water and passed through nylon filters (13 mm, 0.45 μm). The remaining concentrations of EGCG or its glucoside were analyzed by HPLC.

Trolox Equivalent Antioxidant Capacity (TEAC) Assay

The $ABTS^{\cdot+}$ was generated from ABTS solution (7 mM) with potassium persulfate (2.45 mM) for 15 h. The radical cation absorbed at 734 nm and was stable for 2 days. $ABTS^{\cdot+}$ was diluted in ethanol to 0.7 ± 0.02 absorbance units at 734 nm. Addition of antioxidants to the pre-formed radical cation reduces it to ABTS thus decreasing the absorbance. Twenty microliter of antioxidant solution (between 20 and 210 μ M) was added to 230 μL of adjusted $ABTS^{\cdot+}$ solution. The decrease of absorbance of the $ABTS^{\cdot+}$ solution was monitored at 734 nm during 6 min using a microplate reader (model Versamax, Molecular Devices). The decrease of absorbance was determined measuring the area under the curve. (R)-Trolox was used as a reference antioxidant. The TEAC value was expressed as the concentration (μM) at which the compound decreases the same absorbance as 1 μM (R)-Trolox.

Cell Cultures

SH-S5Y5 neurons were cultured in collagen-pretreated petri-dishes with DMEM-F12 medium supplemented with penicillin/streptomycin and 10% inactivated fetal bovine serum (iFBS). RAW 264.7 macrophages and HT-29 colon cancer cells were cultured in DMEM high glucose medium supplemented with penicillin/streptomycin and 10% iFBS. MRC5 were cultured in DMEM low glucose medium supplemented with glutamine, penicillin/streptomycin and 10% iFBS.

Cell Viability Assays

Neuron assays were done in collagen-pretreated 96 well plates by seeding 2×10^4 neurons per well in a 100 μL volume and with 24 h of incubation before the compound addition. Macrophage assays were done in 96 well plates by seeding 2.5×10^4 macrophages per well in a 100 μL volume with 4 h of incubation before the compound addition. MRC5 and HT-29 assays were done in 96 well plates by seeding 5×10^4 cells per well in a 100 μL volume and with 24 h of incubation before the compound addition. Tested compounds dissolved in DMSO were then added at different final concentrations (100, 10, and 1 μM) to determine compound toxicity. Final DMSO percentage in each cell was adjusted to 1%. Cell viability was evaluated 24 h (SH-SY5Y and RAW 264.7 cells) or 48 h (MRC5 and HT-29 cells) after compounds addition by mitochondrial MTT assay, according to manufacturer.

Measurement of Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) levels were evaluated using the ROS-sensitive H_2DCFDA staining method (Sigma, St. Louis, MO, USA). The intracellular ROS level was determined on SH-SY5Y neuroblastoma cells that were cultured, plated and compound-treated as described previously for the cell viability assay. The protective effect of the EGCG derivatives on H_2O_2-induced oxidative stress was assayed after a short pre-incubation time of the compounds (2 h) followed by a short incubation with H_2O_2 (100 μM, 2 h). The intracellular ROS generation of each compound alone, without H_2O_2 treatment, after 6 h

of incubation, was also evaluated. Following treatments, the medium was removed and incubated with 25 μM H_2DCFDA for 2 h at 37°C in the dark. H_2DCFDA, a cell permeable non-fluorescent, is de-esterified intracellularly and turns to the highly fluorescent permeant molecule 2,7-dichlorofluorescein (DCF) in the presence of intracellular ROS upon oxidation. Fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a multimode microplate reader (TECAN, Männedorf, Switzerland).

Mitochondrial Oxidation–Reduction (REDOX) Activity

The analysis of REDOX activity was performed using the fluorogenic oxidation-reduction indicator Resazurin (Life Techonologies Inc., Rockville, MD, USA). The REDOX activity level was determined on SH-SY5Y cells that were cultured, plated and compound-treated as described previously. After treatments, resazurin dissolved in water at a final concentration of 5 μM was added to the wells, and the fluorescence intensity was examined at an excitation of 530 nm and an emission of 590 nm. The plate was incubated for 2 h, and then fluorescence was measured using a multimode microplate reader (TECAN, Männedorf, Switzerland).

Neuroprotective Properties

EGCG and the corresponding glucosides were assayed *in vitro* in cell cultures to determine their neuroprotective activity. SH-S5Y5 neurons were determined on SH-SY5Y cells that were cultured, plated and compound-treated as described previously. EGCG and its glucosides dissolved in DMSO were added at three concentrations (1, 10, and 100 μM) and incubated for 10 min before the addition of hydrogen peroxide (100 μM). Cell viability was evaluated 24 h after compound addition by mitochondrial MTT assay. Neuron recovery was calculated by normalizing the results from H_2O_2-neuron viability to the H_2O_2 positive control.

Statistical Analysis

For the determination of antioxidant capacity (TEAC assay), experiments were performed in triplicate. The standard deviations of TEAC values were calculated from the slope of linear regressions of the curves representing decrease of absorbance vs. concentration. The significant differences between the values were calculated with a *t*-test of slopes and their standard deviations, considering *n* the number of linear regression points.

For the cell viability assays, analysis of ROS, mitochondrial oxidation-reduction activity and neuroprotective activity, averages and standard deviations of at least eight different readings from various experiments were calculated. Welch's *t*-test for samples with unequal variance (previously tested by one way ANOVA in SigmaPlot 13.0) was made to perform the statistical analysis, considering significant differences when $p < 0.05$.

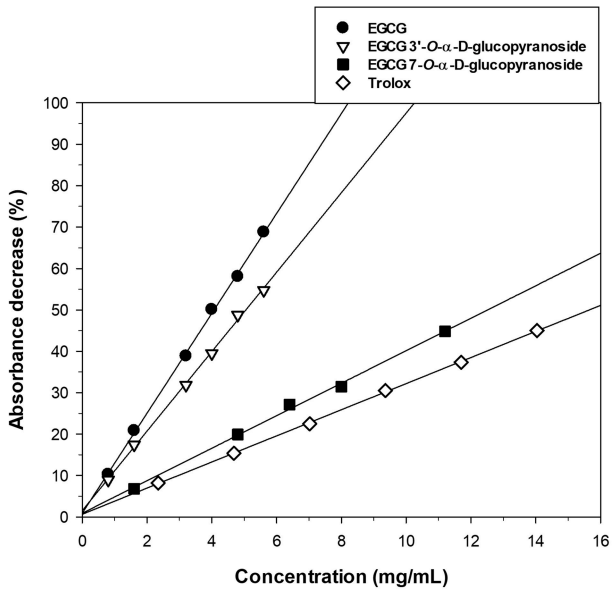

FIGURE 2 | Effect of EGCG and its α-glucosides ***1*** and ***2*** on $ABTS^{\cdot+}$ reduction. In the assay, Trolox was used as antioxidant reference compound.

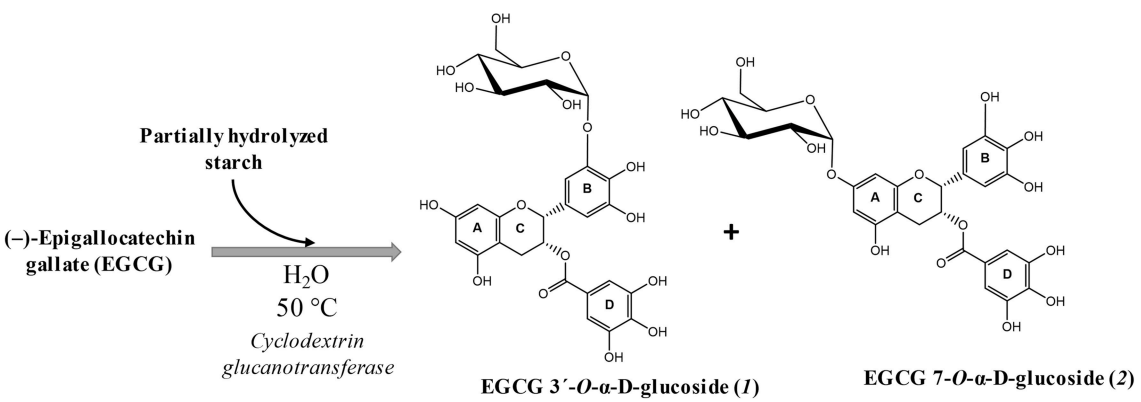

FIGURE 1 | Scheme of the glucosylation of EGCG and the structure of the two main products: epigallocatechin gallate 3′-*O*-α-D-glucopyranoside **(*1*)** and epigallocatechin gallate 7-*O*-α-D-glucopyranoside **(*2*)**.

TABLE 1 | TEAC values of EGCG and its α-glucosides.

Compound	Slope of linear regression	R^2	TEAC
Trolox	3.22 ± 0.02	0.996	1.00 ± 0.02
EGCG	12.1 ± 0.1	0.999	0.27 ± 0.02
EGCG 3′-*O*-α-D-glucopyranoside	9.97 ± 0.20	0.996	0.32 ± 0.02*
EGCG 7-*O*-α-D-glucopyranoside	4.04 ± 0.20	0.999	0.80 ± 0.04*

*The data is expressed as mean ± SD (n = 6, *p < 0.01 vs. EGCG).*

RESULTS AND DISCUSSION

EGCG Glucosylation and Effect on Antioxidant Properties

The synthesis of various α-glucosyl derivatives of (−)-epigallocatechin gallate (EGCG) was performed following a previous work developed in our laboratory (40). The reaction takes place at 50°C catalyzed by cyclodextrin glucanotransferase (CGTase) from *Thermoanaerobacter* sp., using hydrolyzed potato starch as glucosyl donor (**Figure 1**). The reaction was performed in water (no buffer), as the maximum stability of EGCG was found in this solvent (40). Two main monoglucosides were the main products and were chemically characterized by combining MS 2D-NMR methods. The major derivative was epigallocatechin gallate 3′-O-α-D-glucopyranoside (***1***) and the minor epigallocatechin gallate 7-O-α-D-glucopyranoside (***2***).

We studied the antioxidant activity of the two glucosylated derivatives by the TEAC assay to assess the role of the different phenolic groups on the EGCG properties. The results of the assay are represented in **Figure 2**. The incorporation of a α-glucosyl moiety to the position 7 of A-ring caused a higher loss of antioxidant activity than in position 3′ of B-ring. The TEAC values, calculated from the slopes of linear regressions of **Figure 2**, are summarized in **Table 1**. In all cases the TEAC values were lower than that obtained for Trolox.

As shown in **Figure 2**, the glucosylation at the 3′-position has a slight influence on the scavenging activity of EGCG toward $ABTS^{\cdot+}$ radicals. In this context, it has been reported that the *ortho*-trihydroxyl group (at positions C-3′,−4′, and−5′) at B-ring and the gallate moiety at C-3 of A-ring are the most important structural features for scavenging free radicals by EGCG (38, 41). Our results compare well with those described by Nanjo et al. using the DPPH radicals assay (41, 42) However, it must be considered that the free radical scavenging capacity of tea catechins and their derivatives is radical-dependent (32). In the case of DPPH radical scavenging, it has been demonstrated that both the 4′-OH at B-ring and the 4″-OH at the galloyl moiety are essential to maintain antioxidant activity (39, 43).

Stability of EGCG Glucosides

It is well-reported that the stability of EGCG in aqueous solutions is rather limited (37, 44, 45). The two main processes involved in the degradation of EGCG are epimerization and oxidative coupling (34). The stability of EGCG is concentration-dependent and can be also influenced by temperature, pH and the amount of oxygen in the solution, among other parameters (32).

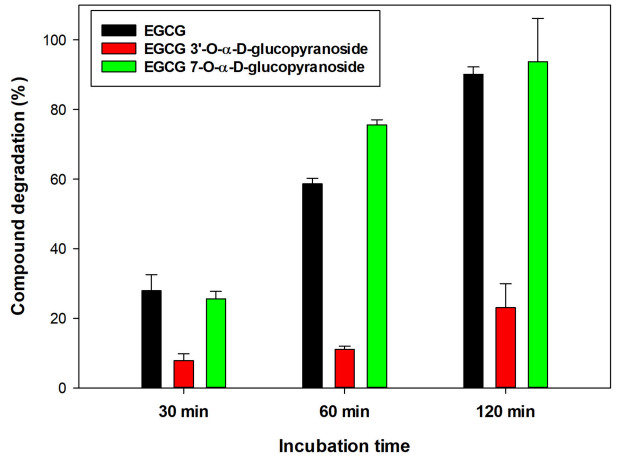

FIGURE 3 | Relative degradation of EGCG and its α-glucosides ***1*** and ***2*** under standard conditions: [Compound] = 4 mg/ml, 20 mM sodium phosphate buffer (pH 6.7), 60°C.

The stability of EGCG and its two monoglucosides in a buffered solution was comparatively studied. The compounds (4 mg/mL) were dissolved in 20 mM phosphate buffer (pH 6.7) and incubated at 60°C. As shown in **Figure 3**, the EGCG was degraded about 4-fold faster than the monoglucoside ***1***. The degradation process was concomitant with the appearance of (−)-gallocatechin gallate (GCG) as a result of EGCG epimerization (data not shown). The color of the solutions became brown upon incubation, as a consequence of the formation by oxidative coupling of dimers and compounds of higher molecular-weight (46).

After 1 h incubation, 59% of initial EGCG and 76% of monoglucoside ***2*** had disappeared, in contrast with only 11% of the monoglucoside at 3′-OH. In this context, Noguchi et al. reported that the 5-*O*-α-D-glucopyranoside of EGCG was about 1.5-fold more stable than the parent compound at pH 7.0 and 80°C (36). Kitao et al. reported that the α-monoglucoside at C-4′ of B-ring was also substantially more stable than EGCG (37). Therefore, the glycosylation of EGCG in position 3′ of B-ring increases significantly the resistance of EGCG to pH and thermal degradation.

Toxicity of EGCG Glucosides

The toxicity of EGCG and the isolated monoglucosides ***1*** and ***2*** was tested in four cell lines (human SH-S5Y5 neurons, RAW 264.7 macrophages, MRC5 fibroblasts and HT-29 colon cancer cells). The viability of cells in the presence of the compounds was determined at three concentrations (1, 10, and 100 μM). The final DMSO percentage in each cell was adjusted to 1% (v/v). The values were referred to the control (cells containing 1% DMSO). As shown in **Figure 4**, EGCG and its glucosides were not significantly toxic for any of the examined cell lines, except for the parent compound EGCG at 100 μM concentration in HT-29 colon cancer cells (**Figure 4D**).

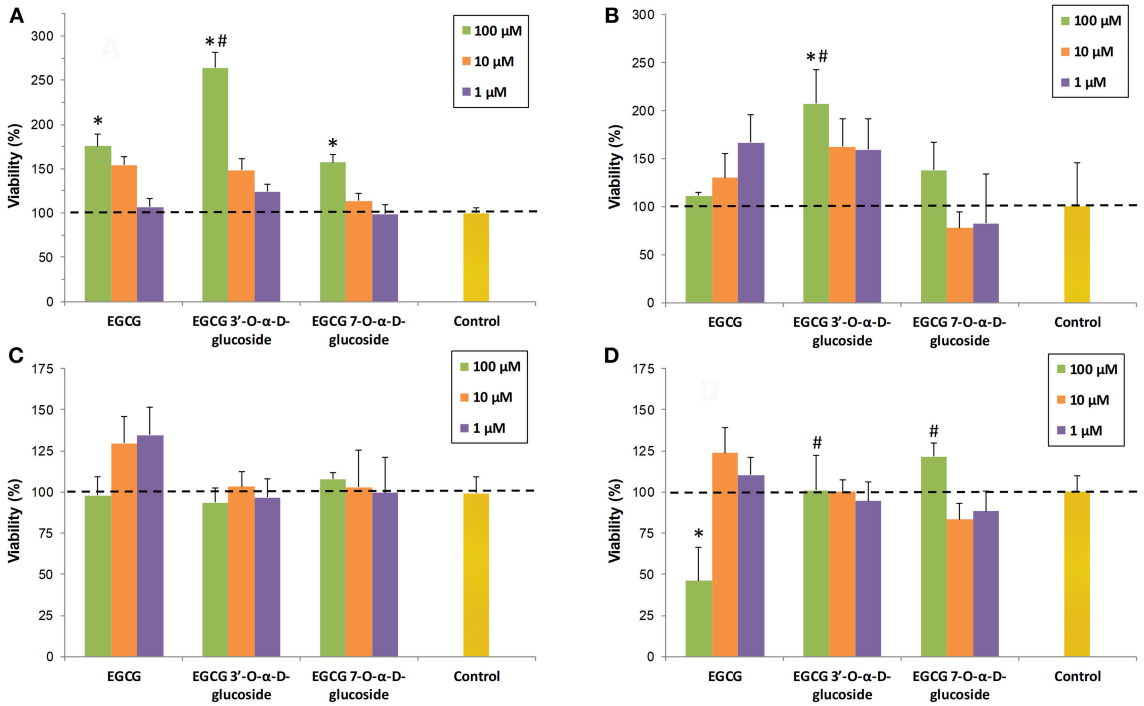

FIGURE 4 | Cell viability assays in presence of EGCG and its α-glucosides ***1*** and ***2*** on: **(A)** SH-SY5Y neuronal cells; **(B)** RAW 264.7 macrophages; **(C)** MRC5 fibroblasts; **(D)** HT-29 colon cancer cells. The values are referred to the control (cells containing 1% DMSO). The data is expressed as mean ± SD ($n = 8$, $^*p < 0.05$ vs. control group; $\#p < 0.05$ vs. EGCG (100 µM) group).

The cytotoxic effect on HT-29 cancer cells correlates well with previous reports on the specific pro-oxidant action of catechins toward cancer cells (47), which seems to be modulated by sirtuin 3 (SIRT3) (48). Thus, green tea catechins (including EGCG) may exert pro-oxidant activity in cancer cells leading to cell death but antioxidant effects in normal cells (49).

We have observed that the presence of a glucose unit in a natural phenolic compound such as resveratrol, like in piceid (3-β-glucoside of resveratrol), also decreases the intrinsic toxicity of the parent molecule in human embryonic kidney cells (HEK-293) (21). However, this is not a general trend since piceid is more toxic than resveratrol for HT-29 and breast adenocarcinoma MCF-7 cancer cells. The differences in cellular uptake of the compounds could be related to the observed toxicity, especially if the glucose transporters are playing a role in the entrance of the glucoside derivatives.

ROS and REDOX Activity of EGCG Glucosides

Once established the safety of EGCG and EGCG glucosides toward SH-SY5Y neuroblastoma cultures, their potential to alleviate intracellular ROS levels or to boost intracellular REDOX activity was determined (50). The former assays were carried out in the presence of hydrogen peroxide as intracellular ROS trigger. Basal ROS levels (**Figure 5A1**) were measured from the fluorescence intensity of DCF as it is explained in the Experimental section. As a rule, all compounds produced a dose-response decrease in ROS levels, but this effect was significantly greater for EGCG and EGCG 3′-*O*-α-D-glucoside, compared to EGCG 7-*O*-α-D-glucoside (**Figure 5A2**). Remarkably, the treatment with 100 µM EGCG and its 3′-α-D-glucoside lowered ROS levels to nearly 50% of the non-stimulated cells value.

Regarding REDOX activity, H_2O_2 treatment led to a small decrease of REDOX compared to control cells, which was attenuated by a 100 µM pretreatment with all the compounds screened (**Figure 5B2**). Bigger differences were observed in REDOX activity between control cells and pretreatment with each compound alone for 6 h, where all the derivatives at 100 µM were able to increase the basal REDOX activity regardless the treatment concentration (**Figure 5B1**).

Neuroprotective Activity of EGCG Glucosides

EGCG has arisen a lot of interest as a potential therapeutic agent in the prevention of neurodegenerative diseases (51–53). This ability is related with its antioxidant, radical scavenging, anti-apoptotic and anti-inflammatory properties (54). Several studies confirmed the potential of EGCG to promote healthy aging, suppress cognitive dysfunction, increase learning ability and minimize oxidative damage in the brain (55, 56).

In the present work, the neuroprotective activity of EGCG and the synthesized monoglucosides ***1*** and ***2*** toward human SH-S5Y5 neurons was tested *in vitro*. Previously we demonstrated that EGCG and its glucosides were not toxic for the cells (**Figure 4A**). Then, the neuroprotective activity in the presence of H_2O_2 was tested at the same compound concentrations (1, 10, and 100 µM)

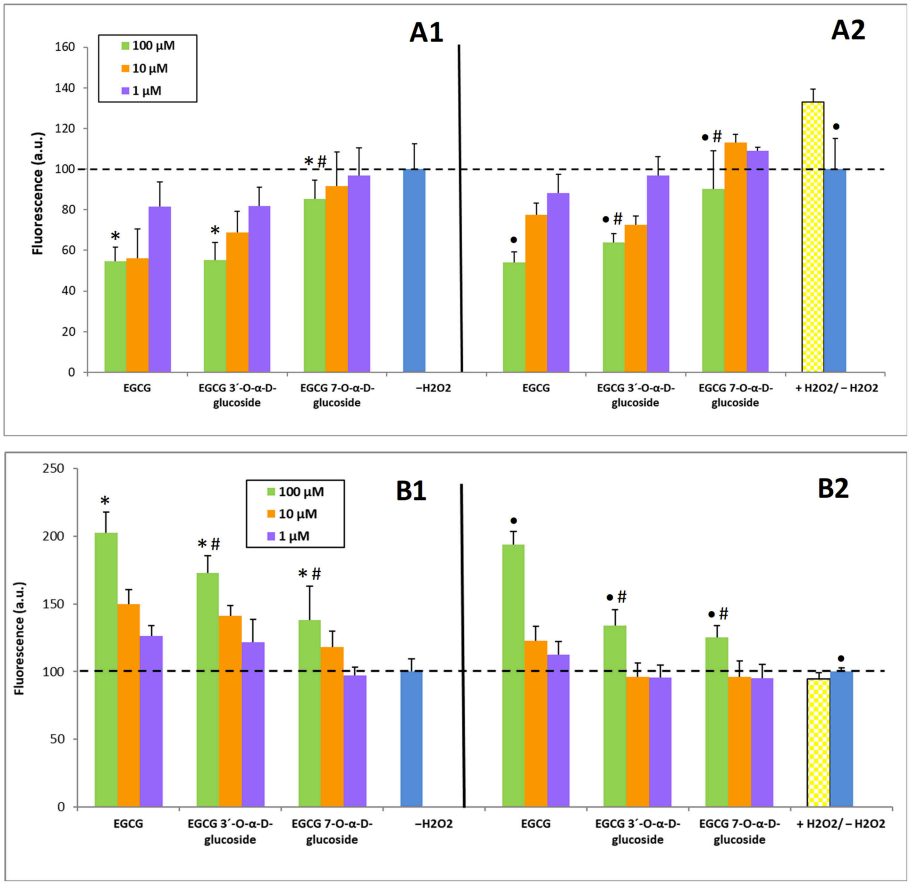

FIGURE 5 | Capacity of EGCG and its α-glucosides ***1*** and ***2*** on SH-SY5Y neuronal cells to: **(A)** Alleviate intracellular ROS levels; **(B)** Enhance intracellular REDOX activity. **(A1, B1)** Incubation (6 h) with the compounds without H_2O_2 treatment; **(A2, B2)** Pre-incubation (2 h) with the compounds followed by incubation (2 h) with 100 µM H_2O_2. The values are normalized to the experiments in absence of H_2O_2 ($-H_2O_2$). The data is expressed as mean ± SD ($n = 8$, $^*p < 0.05$ vs. $-H_2O_2$ group; $^\bullet p <$ 0.05 vs. $+H_2O_2$ group; #p < 0.05 vs. EGCG (100 µM) group).

(**Figure 6**). Values above 100% indicated neuroprotection**.** EGCG and its glucoside ***1*** showed a dose-dependent behavior increasing cells viability after exposure to hydrogen peroxide. In particular, the viability increased 2.75-fold, referred to the cells treated with H_2O_2, in the presence of 100 µM of the 3′-glucoside, whilst EGCG increased 1.7-fold the viability of cells. This increased neuroprotection of monoglucoside ***1*** compared to EGCG might be related with their similar antioxidant activity (**Figure 2**) but the slower degradation of the 3′-glucoside (**Figure 3**). The enhancement of neuroprotective activity upon glycosylation was more significant than the reported with other related polyphenols such as hydroxytyrosol (13).

Both EGCG and its 3′-α-D-glucoside exhibited better properties at 100 µM than the α-glucoside at C-7 of the A-ring (compound ***2***). This result could be related with the lower antioxidant activity of the C-7 monoglucoside compared with EGCG and its derivative at C-3′ (**Figure 2**). In this context, Xiao recently reported that several polyphenols with catechol or pyrogallol structure were unstable in cell culture medium such as DMEM in the absence of cells (57). For that reason, the different stability of EGCG and its glucosides (**Figure 3**), and in particular the stabilization effect upon glycosylation at C-3′, could play a critical role in the bioactivity results presented in this work.

CONCLUSION

Two α-glucosides of EGCG were enzymatically synthesized and their properties assayed. The major product ***1*** contained a glucosyl moiety at C-3′ in the B-ring and the minor compound ***2*** was glucosylated at C-7 of A-ring. The compound ***1*** exhibited more interesting properties than ***2***. Thus, it displayed higher pH and thermal stability than EGCG, and a similar radical scavenging activity. It is remarkable that the viability of H_2O_2-treated human neurons increased 2.75-fold in the presence of monoglucoside ***1***, whilst EGCG only produced a 1.7-fold enhancement. In conclusion, the α-glucoside of EGCG at C-3′ could be useful for nutraceutical, cosmetic and biomedical applications. However, to determine its full potential, further studies regarding the bioavailability and *in vivo* activity are necessary.

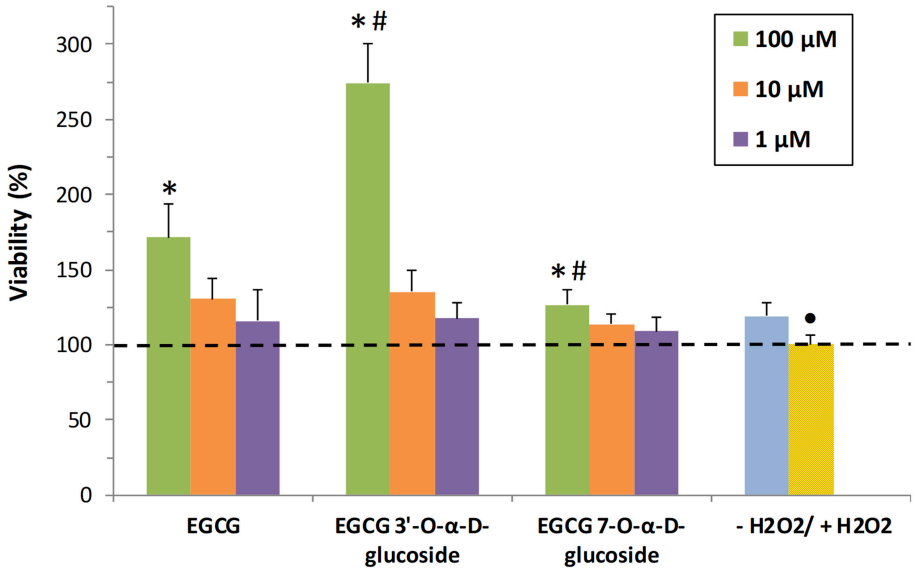

FIGURE 6 | *In vitro* analysis of neuroprotective activity of EGCG and its α-glucosides ***1*** and ***2*** on SH-SY5Y neuronal cells. The values are referred to the viability of cells in presence of H_2O_2 ($+H_2O_2$). The data is expressed as mean ± SD ($n = 8$, $^*p <$ 0.05 vs. $+H_2O_2$ group; •$p < 0.05$ vs. $-H_2O_2$ group; #$p < 0.05$ vs. EGCG (100 µM) group).

AUTHOR CONTRIBUTIONS

FP, JM, and AB conceived and designed the experiments. JG-A and PP performed most of the experiments. FP and JM wrote the paper, which was improved by the rest of authors.

FUNDING

This work was supported by a grant from the Spanish Ministry of Economy and Competitiveness (BIO2016-76601-C3-1-R).

ACKNOWLEDGMENTS

We thank Ramiro Martinez (Novozymes A/S) for supplying Toruzyme and for critical suggestions. We thank the support of COST-Action CM1303 on Systems Biocatalysts. We acknowledge support of the publication fee by the CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI).

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Conflict of Interest Statement: The 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.

frontiers in Nutrition

ORIGINAL RESEARCH
published: 12 March 2019
doi: 10.3389/fnut.2019.00023

Metabolism of Milk Oligosaccharides in Preterm Pigs Sensitive to Necrotizing Enterocolitis

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OPEN ACCESS

Edited by:
F. Javier Moreno, Instituto de Investigación en Ciencias de la Alimentación (CIAL), Spain

Reviewed by:
Tadasu Urashima, Obihiro University of Agriculture and Veterinary Medicine, Japan
Lorena Ruiz, Institute of Dairy Products of Asturias (IPLA), Spain

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Specialty section:
This article was submitted to Nutrition and Food Science Technology, a section of the journal Frontiers in Nutrition

Received: *23 November 2018*
Accepted: *18 February 2019*
Published: *12 March 2019*

Citation:
Rudloff S, Kuntz S, Ostenfeldt Rasmussen S, Roggenbuck M, Sprenger N, Kunz C, Sangild PT and Brandt Bering S (2019) Metabolism of Milk Oligosaccharides in Preterm Pigs Sensitive to Necrotizing Enterocolitis. Front. Nutr. 6:23. doi: 10.3389/fnut.2019.00023

Human milk oligosaccharides (HMO) are major components of breast milk that may have local effects in the gastrointestinal tract and systemic functions after being absorbed, both depending on their metabolism. Using preterm pigs, we investigated the metabolic fate of HMO in three experiments with two different HMO blends. In addition, we examined effects on the colonic microbiota in the presence or absence of necrotizing enterocolitis (NEC). Thus, preterm pigs (n = 112) were fed formula without or with HMO supplementation (5–10) g/L of a mixture of 4 (4-HMO) or >25 HMO (25-HMO) for 5 (Experiment 1 and 2) or 11 days (Experiment 3). Individual HMO were quantified in colon contents and urine using MALDI-TOF-MS (matrix-assisted laser desorption ionization mass spectrometry) and HPAEC-PAD (high-performance anion-exchange chromatography with pulsed amperometric detection). Microbial colonization was analyzed by sequencing of 16S rRNA gene tags. Intestinal permeability was measured by lactulose to mannitol ratio in urine. HMO supplemented to formula were detected in urine and colon contents in preterm piglets after 5 and 11 days in all three experiments. The amount of HMO excreted via the gut or the kidneys showed large individual variations. Microbial diversity in the colon changed from high levels of *Firmicutes* (dominated by *Clostridium*) at day 5 (Exp 2) to high levels of *Proteobacteria* dominated by *Helicobacter* and *Campylobacter* at day 11 (Exp 3). Colonic microbiota composition as well as HMO excretion pattern varied greatly among piglets. Interestingly, the 5-day supplementation of the complex 25-HMO blend led to low concentrations of 3-fucosyllactose (FL) and lacto-N-fucopentaose (LNFP) I in colonic contents, indicating a preferred utilization of these two HMO. Although the interpretation of the data from our piglet study is difficult due to the large individual variation, the presence of *Bifidobacteria*, although low in total numbers, was correlated with total HMO contents, and specifically with 2′FL levels in colonic content. However, early supplementation of formula with HMO did not affect NEC incidence.

Keywords: human milk oligosaccharides (HMO), preterm pigs, metabolism, necrotizing enterocolitis (NEC), microbiota, formula

INTRODUCTION

Increasing evidence supports the hypothesis that benefits of human milk for infants are partly explained by the abundance of complex oligosaccharides. Proposed functions concern their interactions with gut microbiota, the prevention of pathogen adhesion to the epithelium, effects on gut maturation or an influence on the developing brain (1–7). So far, the first human trials with one or two single human milk oligosaccharides (HMO), 2′fucosyllactose (FL) or 2′FL plus lacto-N-neo-tetraose (LNnT), in term infants demonstrated that the new formula were safe and lead to growth rates comparable to those found in term human milk-fed infants (8, 9). Within this context, metabolic aspects of HMO are an important issue as, for example, currently discussed systemic effects such as an influence on inflammatory processes or on brain functions and activity require the preceding absorption of HMO. Indeed, HMO have been detected in the circulation of breast-fed infants (10, 11). To investigate metabolic pathways of single HMOs, studies in infants are limited due to the low HMO availability and also for ethical concerns. Therefore, the selection of an appropriate alternative animal model is an important point to consider. In animals, it has been shown that some HMO can be absorbed (12, 13). As rats, however, do not seem to be suitable for HMO metabolic studies, we used pigs which may have a translational advantage based on the physiological similarity between pigs and humans with regard to the gastrointestinal tract, its delayed maturation and high natural sensitivity to necrotizing enterocolitis (NEC) after preterm birth (14, 15).

In preterm infants, NEC is a major cause of morbidity and mortality affecting 5–10% of infants <1,500 g with a mortality of 20–30% (16, 17). Breast-fed infants were shown to have lower NEC incidences than formula-fed infants (18). In rat pups, three studies showed that the addition of HMO such as disialyl-lacto-N-tetraose (DSLNT), 2′FL or 2′FL plus sialylated galactooligosaccharides, the latter not being present in human milk, decreased NEC incidence (19–21). A potential mechanism for the observed effects may be the upregulation of mucins, and concomitant decrease in intestinal permeability which has recently been shown by pooled HMO (22).

We hypothesized that HMO supplementation of formula leads to fecal and/or urinary excretion of intact HMO at various amounts depending on the prevailing microbiota. If so, not only gastrointestinal but also systemic functions are to be expected. In addition, we aimed at investigating whether HMO supplemented formula affect bacterial colonization and thereby improve NEC resistance in preterm pigs. Because of the potential for synergistic effects among different HMO, we investigated for the first time effects of a large range of HMO, e.g., mixtures containing either 4 or >25 HMO, the latter reflecting the complex oligosaccharide composition in human milk. Clinical and physiological effects on the gastrointestinal tract have been reported previously (23). Here, we focus on HMO metabolism and its potential relations to bacterial gut colonization.

MATERIALS AND METHODS

Information on animal housing is given in the preceeding paper (19). The HMO blends were provided by Glycom A/S (Lyngby, Denmark). The study was approved by the Danish National Committee on Animal Experimentation (license number 2012-15-2934-00193).

Experimental Design and Sample Collection

Experiment 1, 2, and *3* were carried out in preterm born pigs (delivered by Cesarean section at ∼90% of gestation) using different blends of HMO and varying length of exposure time (5 or 11 days postpartum) as described previously (**Figure 1**) (23). HMO blends were chosen to either represent the most abundant individual structures in human milk (4-HMO) or to additionally cover more closely the complex range of oligosaccharides known for human milk with more than 25 compounds present (25-HMO) (**Table 1**).

Preterm pigs ($n = 112$) in each of the three experiments were divided into two groups (control and HMO-treated) based on birth weight and gender (**Figure 1**) The macronutrient content of the diets is described in **Table 2**.

Dietary treatments as well as laboratory analyses were blinded to investigators and all animal procedures (23). In *Experiment 1* and *2*, preterm pigs were fed standard formula with or without HMO for 5 days. In *Experiment 1* ($n = 44$), 5 g/L 4-HMO or maltodextrin (controls) were added to the formula, whereas in *Experiment 2* ($n = 38$), 7 g/L 25-HMO were supplemented to account for the lower abundance of the major HMO used in the 4-HMO blend (23); in *Experiment 3* (11 days, $n = 30$), preterm pigs were fed with 10 and 5 g/L 4-HMO for the first 4 days and up to 11 days, respectively; the control group received ready-to-feed infant formula (RTF-IF, controls) without HMO supplementation. The infant formula was chosen to include a more translational aspect into the longer term experiment. At the end of the experiments, pigs were anesthetized (Zoletil 50, zolazepam/tiletamin; Boehringer Ingelheim, Copenhagen, Denmark), euthanized by injection with sodium pentobarbital, and biological material was collected. Colonic content and urine taken from the bladder were snap-frozen for determination of HMO, colonic microbiota composition, and intestinal permeability. Tissue samples were collected for evaluation of NEC as reported previously (19). Collection of colonic contents and urine could not be achieved from all animals in amounts necessary for all analyses; thus, the number of samples analyzed is given in the corresponding figures.

Evaluation of NEC and Intestinal Permeability

Stomach, small intestine (proximal, middle, and distal regions), and colon were evaluated for NEC lesions and given a score from 1 (absence of macroscopic lesions), 2 (local hyperemia), 3 (hyperemia, milch hemorrhage, extensive edema), 4 (extensive hemorrhage), 5 (local necrosis, and pneumatosis intestinalis) to 6 (extensive necrosis and intramural gas cysts). NEC was

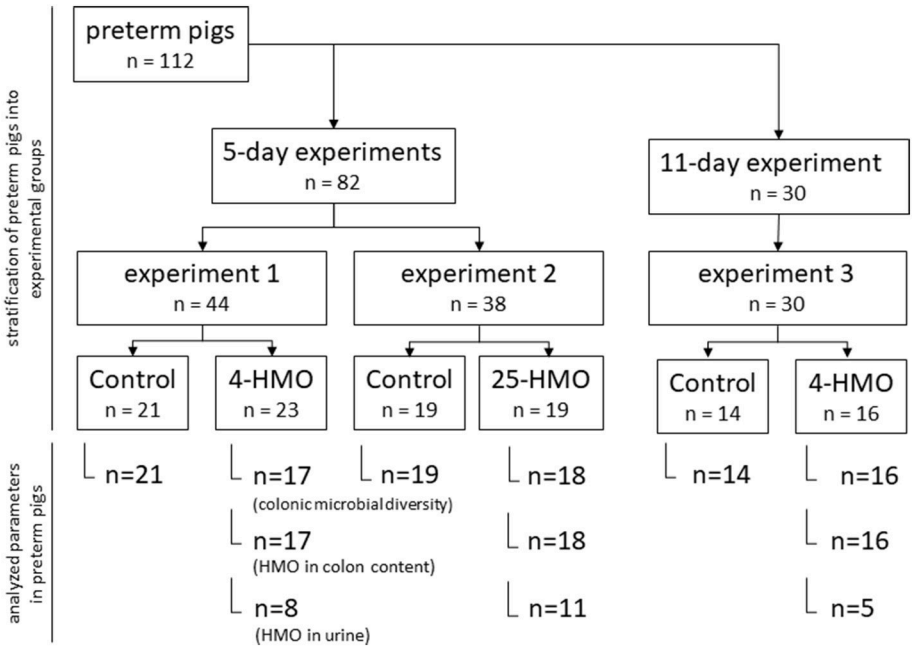

FIGURE 1 | Experimental design for 5-day and 11-day experiments in preterm pigs.

defined as a score of ≥3 in any of the evaluated regions (23). Intestinal permeability was determined by the lactulose to mannitol ratio in urine. Pigs were fed 5% lactulose and 5% mannitol (15 mL/kg body weight) 3 h prior to euthanasia, and were fed half a bolus of their respective diets 1.5 h prior to euthanasia. Urine was collected at euthanasia, and lactulose and mannitol concentrations were analyzed spectrophotometrically as described previously (24).

Analytical Procedures for HMO Quantification

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was applied for discrimination of stereoisomeric HMO, e.g., lacto-N-tetraose (LNT) and LNnT as well as for identification and quantification of HMO in urine and colonic contents using external standard oligosaccharides (Carbosynth Ltd, Berkshire, UK; Dextra, Reading, UK; Elicityl, Crolles, France) (25, 26). To verify the presence of HMO determined by HPAEC-PAD, matrix-assisted laser desorption ionization mass spectrometry (MALDI-TOF-MS) was used as described (26). Briefly, samples (urine and solubilized colon contents) were centrifuged after the addition of pure water. For normalization of the amount of urinary constituents applied to the extraction procedure, creatinine concentration was determined colorimetrically (R&D Systems, Heidelberg, Germany). Solid phase extraction with porous graphitic carbon cartridges (for colonic contents: HyperSep-96 Wells, 25 mg; for urine: HyperSep Hypercarb 50 mg; Thermo Scientific, Bellefonte PA, USA) was performed via a Hamilton Microlab Starlet liquid handling system (Hamilton Robotics, Reno, NV, USA) or manually for urine samples. The conditions for cartridge equilibration as well as the elution of oligosaccharides have been described previously (25, 26).

After solid phase extraction, oligosaccharides were dried overnight in a vacuum centrifuge and resuspended in water. An HPAEC-PAD system (Dionex ICS-5000) equipped with a CarboPac PA-1 and a guard column was operated using the Chromeleon 6.80 software (ThermoFisher Scientific, Dreieich, Germany). The running parameters at a constant flow rate of 0.5 mL/min were as follows: 0.1 mol/L sodium hydroxide from 0 to 15 min, followed by a linear gradient up to 0.25 mol/L sodium acetate in 0.1 mol/L sodium hydroxide for 87 min. External oligosaccharide standards were used for peak identification and the area under the curves were determined. Individual 4-point calibrations were used for quantification of oligosaccharides in extracts from urine and colonic contents. Mass spectra from the same samples in triplicate determinations were acquired using an Ultraflex I instrument (Bruker Daltonics, Bremen, Germany). Oligosaccharide profiles were acquired in positive-ion mode over a mass range of m/z 340–3,200. Data acquisition and analysis were performed by flexControl and flexAnalysis 3.0 software (Bruker Daltonics, Bremen, Germany), respectively (25, 26).

Identification and Characterization of Microorganisms

Colonic bacterial microbiota composition was determined by tag-encoded 16S rRNA gene MiSeq-based high throughput sequencing (Illumina, San Diego, CA, USA) as published recently (27). Briefly, DNA was extracted from 0.5 g colon content using the PowerSoil DNA Isolation Kit (MoBio Laboratories). The V3-V4 region of the 16S rRNA gene was amplified with the universal

TABLE 1 | HMO compositions in 4-HMO and 25-HMO blends.

No.	Compound[a]	Abbreviation	Amount in 4-HMO (g/100 g blend)	Amount in 25-HMO (g/100 g blend)
1	2′Fucosyllactose	2′FL	61.9	18.7
2	Lacto-N-neo-tetraose	LNnT	10.3	7.9
3	Lacto-N-tetraose	LNT	14.4	7.9
4	6′Sialyllactose	6′SL	10.3	3.6
5	3-Fucosyllactose	3-FL	–	12.1
6	Difucosyllactose	DF-L	–	2.7
7	Lacto-N-fucopentaose I	LNFP I	–	6.5
8	Lacto-N-fucopentaose II	LNFP II	–	2.7
9	Lacto-N-fucopentaose III	LNFP III	–	1.8
10	Sialyllacto-N-tetraose a	LST a	–	0.9
11	Sialyllacto-N-neo-tetraose c	LST c	–	2.7
12	3′Sialyllactose	3′SL	–	3.7
13	Disialyllacto-N-tetraose	DSLNT	–	7.0
14–32	Other oligosaccharides[b]	–	3.1	8.6
	Others (mainly salt)		–	2.9
	TOTAL		**100.0**	**100.0**

The abundances of each HMO are presented as a relative amount of the total measured area under the curve as determined by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) for each HMO.

[a] Compounds 1–13 (90% of all oligosaccharides in the 25-HMO blend).

[b] Compounds 14–32 (not quantified).

TABLE 2 | Composition of the formula used in *Experiment 1–3*.

	Standard Formula[a]	RTF-IF[b]
Energy, kJ/L	4,115	3,400
Protein, g/L	73	29
Fat, g/L	59	40
Carbohydrates, g/L	42	84
Lactose, g/L	-	36
Maltodextrin, g/L	46	45
Oligosaccharides, g/L	–[c]	–[d]

[a] Piglet formula mixed from Pepdite 2-0, Liquigen-MCT (SHS International, Liverpool, UK), and Lacprodan DI-9224 (Arla Food Ingredients, Aarhus, Denmark).

[b] RTF-IF, ready-to-feed infant formula for preterm infants (Alprem, Clinic 1, Nestlé Nutrition S.A., Barcelona, Spain).

[c] Supplemented with maltodextrin (control) or HMO [Experiment 1 (5-day): 5 g/L 4-HMO; Experiment 2 (5-day): 7 g/L 25-HMO].

[d] With and without HMO supplementation [Experiment 3 (11-day): 10 g/L 4-HMO blend during the first 4 days, then 5 g/L as in Experiment 1].

prokaryotic primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′ in a first PCR revealing amplicon lengths of 290 bp (28–30). In a second PCR, adapters compatible with the Nextera Index Kit (Illumina) where attached to the amplicons. After the amplification of fragments with adapters and tags, these were purified and clean constructs were quantified prior to library pooling, by using a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA). The 2 × 250-bp sequencing reaction followed the standard procedure of Illumina MiSeq for pair-end reads. After sequence generation, the reads were demultiplexed and paired followed by a clean-up step to truncate primers, remove low quality sequences, and chimeras following the default settings of the UPARSE pipeline. Operational taxonomic units (OTUs) were picked with USEARCH at >97% sequences identity and classified using Mothur (v.1.33.3) and the RDP database (31, 32). Uneven sequencing depth was corrected using a zero-inflated Gaussian distribution implanted in the R package of MetagenomeSeq (33).

Statistical Analysis

Statistical analyses were carried out using GraphPad Prism 6.0.1.3 (GraphPad Software); results were expressed as medians with IQR (interquartile range, 10th−90th percentiles). D'Agostino Pearson omnibus normality tests were used to determine whether data sets were well-modeled by normal distribution. If necessary, log transformation was used. As indicated, data were analyzed by ANOVA or student *t*-test. Corrections for multiple comparisons were made using the Holm-Sidak method. Differences were considered significant at $P < 0.05$.

Associations were described by using Spearman's or Pearson correlation coefficients. Correlation analyses were shown as correlation coefficient r with 95% confidence interval.

Taxonomic relative abundance data (OTUs >5%) were used to calculate correlations between HMO consumption and fecal bacteria abundance. A Spearman's correlation was used to describe associations. Differences between groups were assessed using ANOVA and Tukey multiple-comparison test.

RESULTS

Identification and Characterization of HMO

To analyze oligosaccharides in the HMO blends as well as in urine and colonic contents, we applied HPAEC-PAD and MALDI-TOF-MS. In the complex 25-HMO blend **(Table 1)**, 13 HMO

were quantified, which, however, comprised almost 90% of all HMO present (**Figure 2A**). In **Figure 2B**, exemplary MALDI-TOF-MS spectra of urine from pigs fed a control formula or a formula supplemented with 4-HMO are shown. The control pig received a standard formula containing higher saccharides (maltodextrin), which was reflected by hexose oligomers of different lengths. For the pig receiving HMO, the mass-to charge ratio (m/z) of 511, 656, and 730 represent 2′FL, 6′SL, and the isomers LNT and LNnT deriving from the 4-HMO blend.

HMO in Colon Contents and Urine

Five-Day Supplementation of 4-HMO (Experiment 1) or of 25-HMO (Experiment 2)

The total colonic HMO content was 46.9 (20.6–337.7) mg/g dry weight and in urine 16.2 (3.3–48.8) mg/μmol creatinine (**Figure 3A**). Large variations were observed in both the total HMO concentrations and individual components of the 4-HMO blend. There seemed to be no specificity in the HMO degradation or absorption. The 25-HMO blend, designed to be closer to that normally found in human milk than the 4-HMO was used in *Experiment 2*. With the 25-HMO blend, the total colonic HMO content was 9.8 (0–29.1) mg/g dry weight and in urine 56.6 (9.3–127.3) mg/μmol creatinine (**Figure 3B**). As with the 4-HMO blend, supplementation with the 25-HMO blend revealed a large individual variability in HMO excretion via colon contents and urine. In contrast to 2′FL in both HMO blends, 3-FL was metabolized differently. Whereas, the colon contents of 2′FL varied greatly [0.9 (0–6.3) mg/g dry weight], the variation of the 3-FL excretion was small with only low amounts detectable in the colon and urine [0.01 (0–0.9) mg/g dry weight and 0.5 (0–1.1)

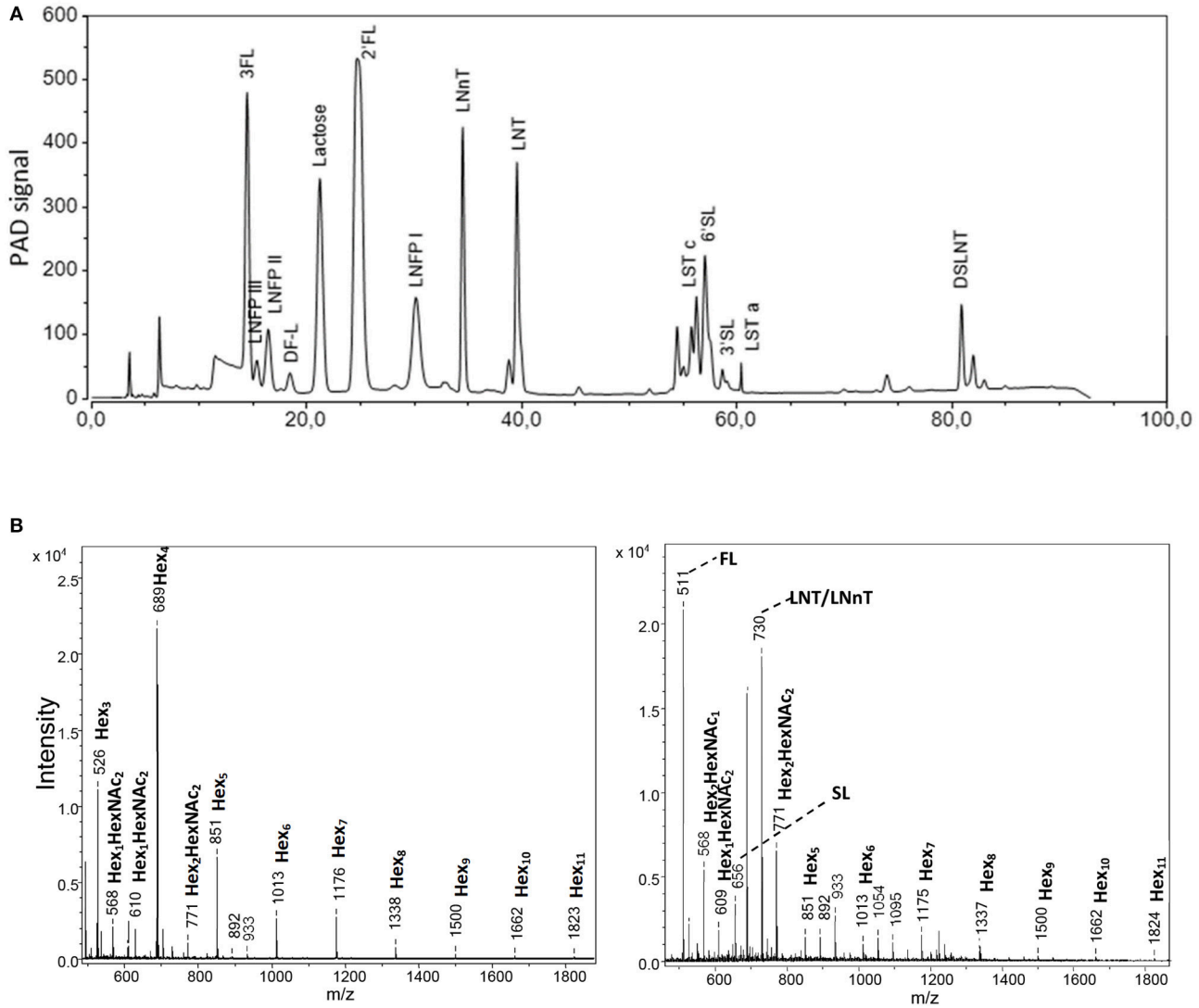

FIGURE 2 | Analytical procedures comprising **(A)** high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). From the complex HMO blend named 25-HMO, 13 compounds were used for quantification (comprise 90% of all oligosaccharides in this blend), and **(B)** matrix-assisted laser desorption ionization mass spectrometry **(**MALDI-TOF-MS). The panels show a representative profile of urine from piglets from *Experiment 1* fed the standard formula without (left panel) and with (right panel) a 4-HMO blend. The mass-to-charge (m/z) values are given as nominal mass for $[M+Na]^+$ ions.

mg/μmol creatinine, respectively], indicating an almost complete fermentation. LNFP I, one of the quantitatively major HMO in the 25-HMO blend, also showed very low concentrations in colon content [0.01 (0–0.09) mg/g dry weight]. Its structural isomers, LNFP II and III, were present in much lower concentrations in the 25-HMO blend, but were detectable in higher amounts in colon contents than LNFP I. Compared to the low amount of 3-FL in both colonic content and urine, LNFP I was low in colonic content but showed a large individual variation in urine.

Eleven-Day Supplementation of the 4-HMO Blend (Experiment 3)

In this 11 days lasting experiment, the HMO content in the colon as well as in urine varied largely with an average of 13.8 (0–160.6) mg/g dry weight and 21.4 (5.8–97.0) mg/μmol creatinine, respectively (**Figure 3C**). Similar to the 5-day experiment, there seemed to be no specificity in HMO metabolism as the same proportions between the 4 HMO were found in colonic content and urine after 11 days, however, some pigs showed higher

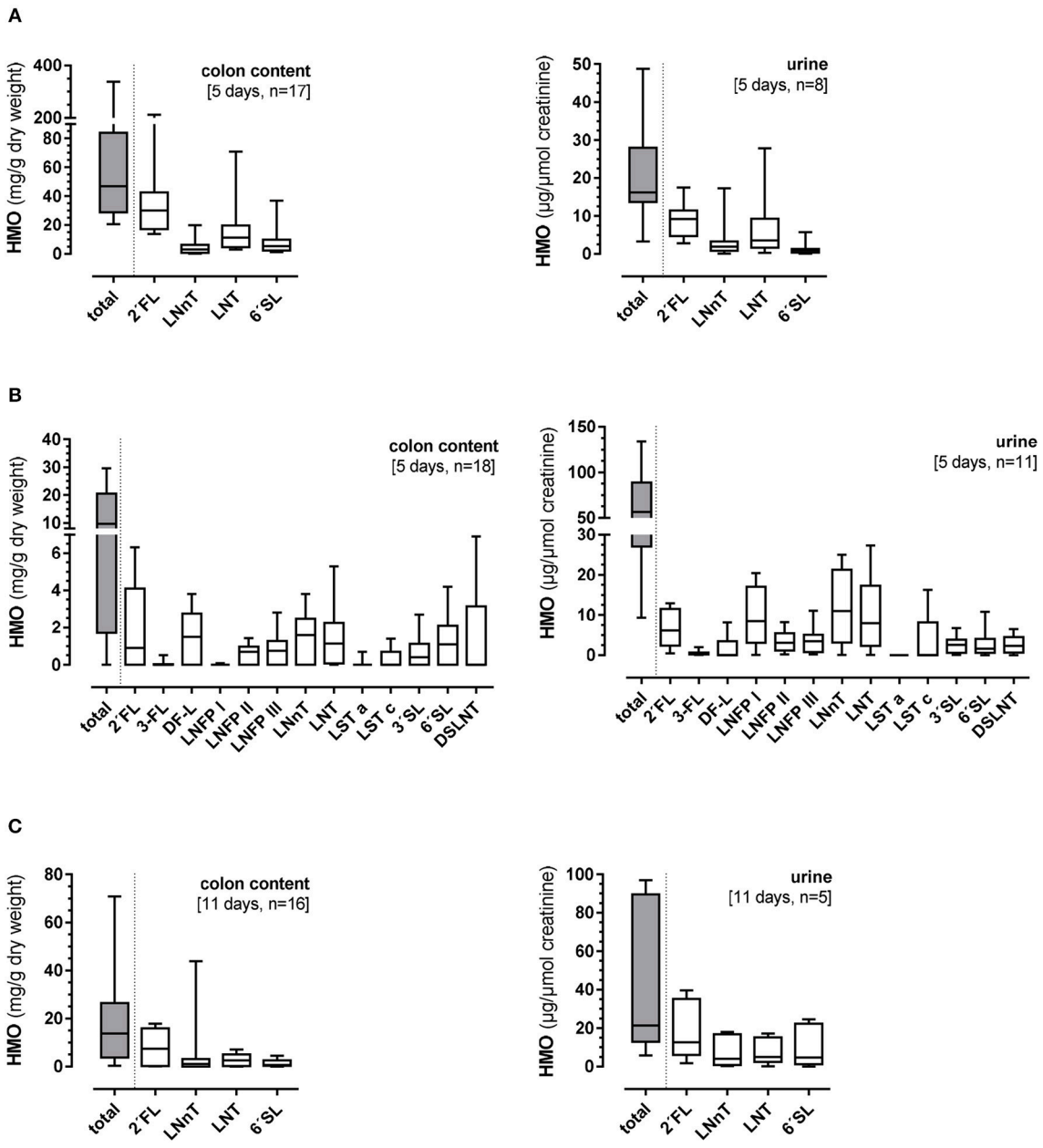

FIGURE 3 | Total and individual concentrations of human milk oligosaccharides (HMO) in colon contents (mg/g dry weight) and urine (mg/μmol creatinine) after **(A)** 5-day supplementation of 4-HMO or **(B)** 5-day supplementation of 25-HMO, and **(C)** after 11-day feeding of 4-HMO. Data are presented as box plots with median and interquartile range (IQR, 10–90th percentiles).

utilization than others. For example, if the concentration of 2′FL was high in colon content and urine, then LNnT, LNT, and 6′SL excretion was high in the same animal (**Figure 4**).

Colonic Microbial Compositions and HMO Concentrations

Figure 5 shows the most prevailing bacterial genera. At the phylum level, only *Firmicutes* were detected in the 5-day experiment when preterm piglets were fed the 4-HMO blend, with *Clostridium, Enterococcus,* and *Lactobacillus* being most abundant (**Figure 5A**). When the complex 25-HMO blend was supplemented within a 5-day period, only few more genera were present. Again, *Clostridium* and *Enterococcus* accounted for more than half of all bacteria (**Figure 5B**).

The 11-day supplementation of the 4-HMO blend revealed an increasingly complex microbial colonization *with Bacteroidetes, Actinobacteria, Proteobacteria, Firmicutes,* and *Fusobacteria* (**Figure 5C**). In contrast to the 5-day experiments, *Proteobacteria* was the most abundant phylum with mainly *Camphylobacter* and *Helicobacter* at the genera level. Furthermore, at the genus level, some *Bifidobacteria* which are well-known for their abilities to consume HMO, were detected in both HMO-fed and control pigs, with only slightly higher proportions in HMO-fed piglets compared to controls ($P < 0.11$). Nevertheless, an inverse correlation was found for *Bifidobacteria* density and total HMO concentrations in colon contents ($P < 0.05$). An even stronger inverse correlation was found for 2′FL, but not for other HMO (**Figure 6**). No correlation was found for *Lactobacillus* and *Bacteroides*.

Correlation of HMO and Cumulative NEC Score and Gut Permeability

Data for clinical outcomes and NEC scores for all 112 pigs have been described previously (23). When comparing HMO levels in the colonic content with the cumulative NEC score, and HMO levels in urine with intestinal permeability, a significant correlation ($r = -0.764$; $P = 0.0033$) was found for HMO in colon content of pigs with NEC in the 5-day experiment receiving the 25-HMO blend. This inverse association was also observed at the individual HMO level for various single oligosaccharides. However, there was no significant correlation between HMO levels in urine and intestinal permeability (**Table 3**).

DISCUSSION

We investigated effects of infant formula supplemented with complex HMO mixtures in a preterm pig model. We documented the metabolism of HMO from blends that either contained the

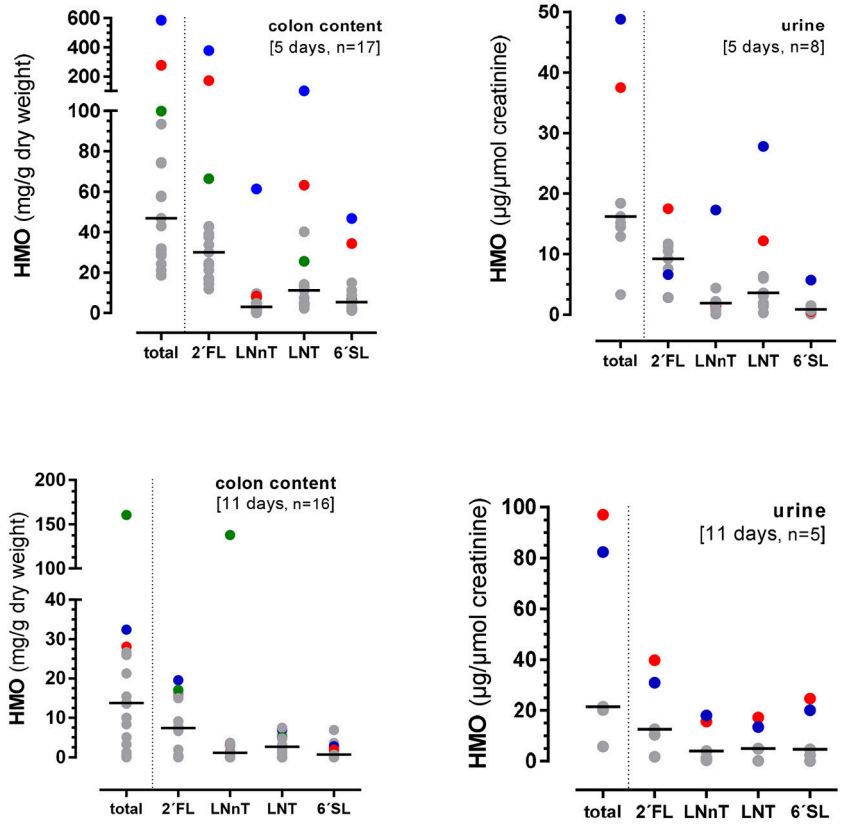

FIGURE 4 | Excretion of total HMOs and individual HMOs from the 4-HMO blend containing 2′FL, LNnT, LNT, and 6′FL via the colon and urine. Different color dots represent HMO concentrations from different pigs with higher HMO excretions.

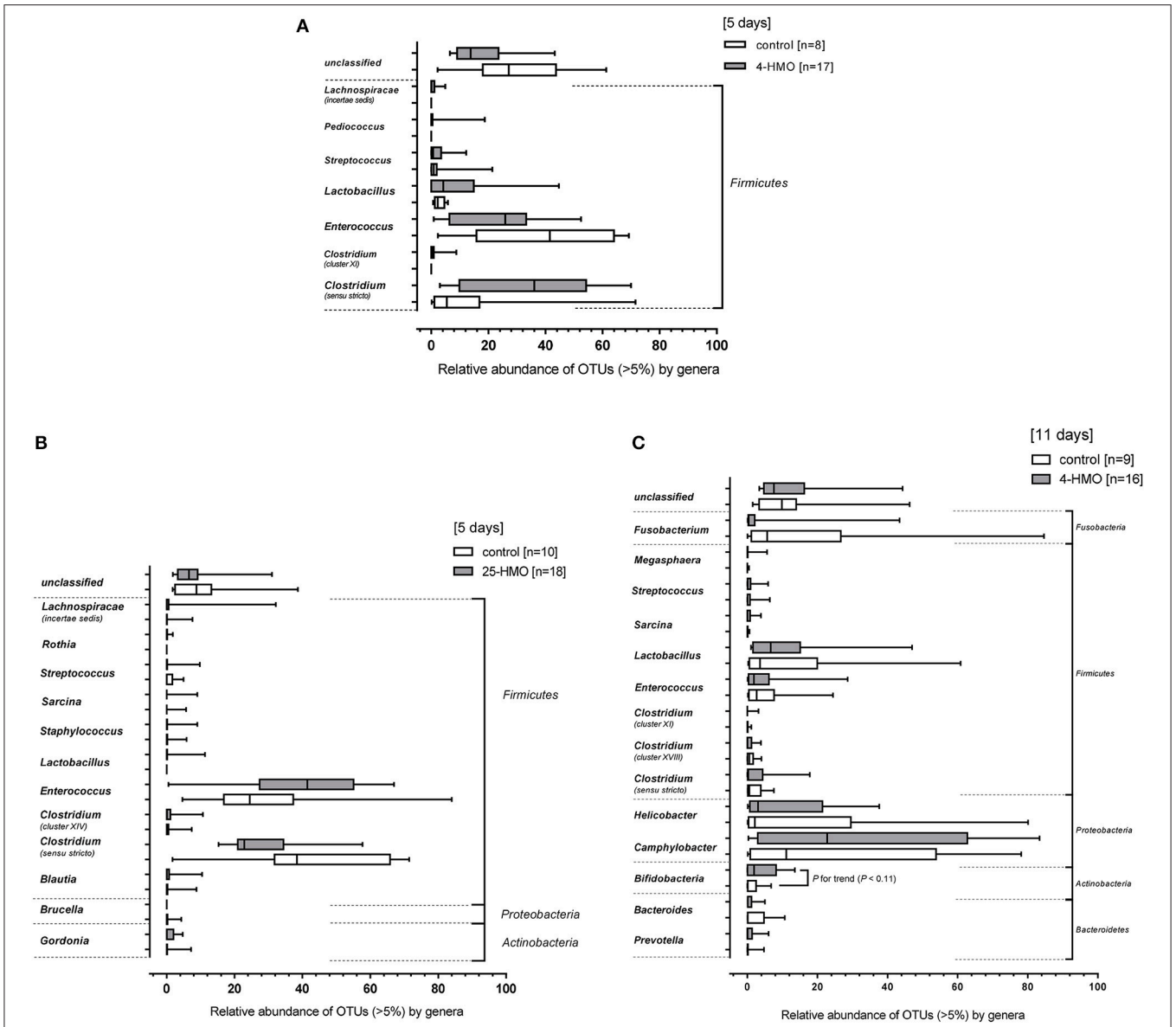

FIGURE 5 | Relative bacterial abundances of taxa in the colonic microbiota at the genus level. Bacterial genera detected in colon content of pigs from **(A)** *Experiment 1* (4-HMO) for (5 days), **(B)** *Experiment 2* (25-HMO for 5 days), and **(C)** *Experiment 3* (4-HMO) for (11 days). Data from control pigs are shown as white bars and those from pigs receiving HMO blends as gray bars. Data are presented as box plots with median and interquartile range (IQR, 10–90th percentiles). Only phyla with relative abundance >5% were included.

major HMO (4-HMO blend) or the most abundant HMO (25-HMO blend) at a ratio of neutral and acidic oligosaccharides of about 80:20 which has been reported for human milk (1). Analyzing urine and feces across three different experiments, we found large individual variations in HMO excretion. This was particularly the case in the 4-days experiment (**Figure 4**) whereas after 11 days this large variation was markedly reduced. Due to the low number of individual samples, it is unclear at the moment whether the excretion is related to the relative distribution of HMO in the formula diet.

For 3-FL and LNFP I, there seems to be a structure-specific metabolism of this single HMO. Their concentration in the colon was very low although they belonged to the major HMO components in the 25-HMO blend. LNFP I is typically found in human milk of secretors (about 70–80% of the population) and shares similar functional epitopes with 2′FL which is currently considered to be a highly potent HMO. 3-FL regularly occurs in human milk, but has until now received limited attention as compared to its structural isomer 2′FL (25). In addition, the linkage of fucose on C-atom 2 of the galactose moiety of 2′FL turns it into a structure with a high potential as an anti-adhesive and anti-inflammatory component. Although the secretor-specific α1-2-fucose linkage is missing in 3-FL, an *in vitro* inhibiton of Norovirus binding has been reported for both

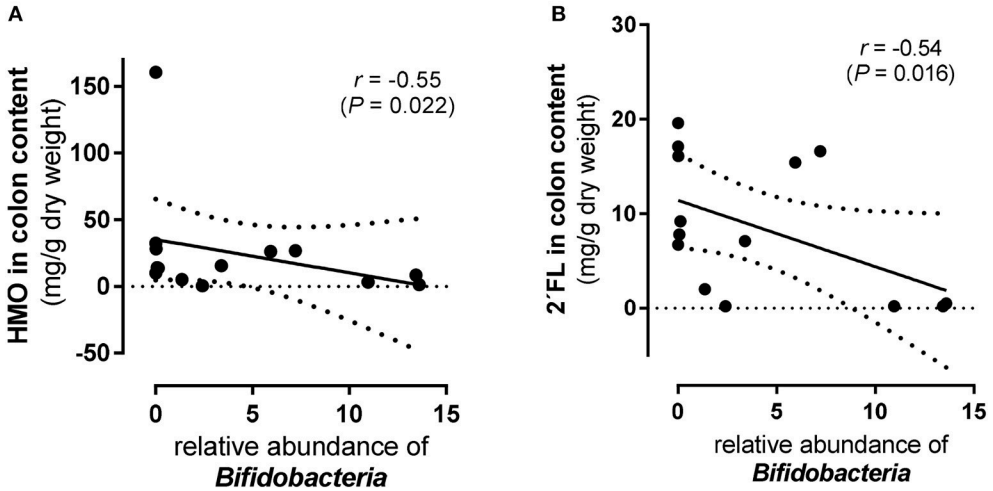

FIGURE 6 | Scatter plots of total and individual human milk oligosaccharides (HMO) and *Bifidobacteria* in colon content from preterm pigs fed the 4-HMO blend for 11 days (*Experiment 3*). Correlation of *Bifidobacteria* abundance with **(A)** total HMO level in colon content and **(B)** with 2′fucosyllactose (FL). Spearman's coefficients (*r*) and *p*-values (*P*) are shown.

TABLE 3 | Correlation of total HMOs in colon and urine with NEC and intestinal permeability, respectively.

No.	*Experiment 1* (5 days, 4-HMO) [piglets, *n*]	*Experiment 2* (5 days, 25-HMO) [piglets, *n*]	*Experiment 3* (11 days, 4HMO) [piglets, *n*]
NEC[a]			
All piglets	−0.318^{nsc} [17]	−0.722^{ns}[18]	−0.316^{ns} [16]
NEC	−0.866^{ns} [4]	−0.764^{**e} [13]	−0.476^{ns} [9]
No NEC	−0.241^{ns} [13]	−0.001^{ns} [5]	−0.722^{ns} [7]
PER[b]			
All piglets	0.121^{ns} [8]	−0.318^{ns} [11]	0.400^{ns} [4]
NEC	n.d.^{d} [2]	−0.866^{ns} [3]	n.d. [2]
No NEC	−0.116 ^{ns} [6]	−0.241^{ns} [8]	n.d. [2]

[a] *Cumulative NEC score: a score of ≥3 in any of the evaluated regions (stomach, proximal, middle, distal small intestine or colon) was defined as NEC.*

[b] *Permeability: lactulose and mannitol contents were analyzed in urine and lactulose to mannitol concentration ratio was used as an indicator of intestinal permeability.*

[c] *Significance was given at $^{**}P < 0.05$ (ns = not significant).*

[d] *n.d. = not determined. The limited sample size hampered detail statistical evaluation for subgroups.*

[e] *Significant correlation for 2′FL ($r = -0.67$; $P = 0.01$), difucosyllactose (DF-L) ($r = -0.69$; $P = 0.008$), LNFP II ($r = -0.72$; $P = 0.0039$), LNT ($r = -0.65$; $P = 0.0076$), LNnT ($r = -0.59$; $P = 0.0011$) and disialyl-LNT (DSLNT) ($r = -0.64$; $P = 0.03$), but not for 3-FL, LNFP I, LNFP III, sialyl-LNT (LST) a, LST b, 3′SL and 6′SL.*

isomers (34). While 3-FL might be primarily utilized by gut microbiota, as it is low in both, colon content and in urine, LNFP I seems to selectively be taken up and excreted in relatively large amounts in urine (**Figure 3B**). Another interesting observation regarding 2′FL is that whenever the fecal excretion of 2′FL was high, the excretion of all other HMOs in the same animal was high as well suggesting an overall low microbial activity.

Regarding disease prevention, the acidic HMO, DSLNT, had remarkable NEC-preventing effects in newborn rats (19). In our preterm pigs, DSLNT was neither clearly related to NEC nor to bacterial colonization despite the high supplementation (about 7% of the 25-HMO blend). These differences could reflect the immaturity of the gut and its bacterial colonization in preterm pigs, a prevailing dysbiosis, which is rather common in cesarean born preterm pigs, with unknown severity, or species-specific differences.

Large individual variability in fecal excretion of HMO have also been found for term and preterm infants fed human milk (26, 35, 36). A gradual change in the fecal oligosaccharide profile in breastfed infants during the first six months postpartum has been reported, without LNT being identified in fecal samples (37). This is in contrast to our previous data (26, 35, 36, 38), where we detected LNT in all fecal samples from human term-born infants whenever HMO were excreted. In infants, there is a large variation in the amount and pattern of HMO excretions, ranging from large amounts to no excretion at all. The reason for this divergence is not known but may be related to a different gut microbial composition and, hence, a different HMO metabolism in the intestinal lumen. For example, LNT, the major core structure of HMO, was initially considered to be a unique growth promoter of *Bifidobacterium longum subsp. infantis* although others, e.g., *Bifidobacteria breve* also use intact LNT (39, 40). In future studies, it is important to relate HMO metabolism in the gut lumen to subspecies level. In the present study we observed that microbial colonization in the two 5-day experiments was limited to *Firmicutes* (Experiment 1) and relatively low numbers of *Actinobacteria* and *Bacteriodetes* (Experiment 2), consisting mainly of *Clostridium (cluster I)* and *Enterococcus*. In Experiment 3 (11-day supplementation of the 4-HMO blend), more bacterial phyla were found, with *Proteobacteria* (mainly *Campylobacter* and *Helicobacter*) as the predominant phylum. *Bifidobacteria* were also detected but in lower abundance. There were no changes in the microbial colonization of the pigs when supplementing formula with the 4-HMO blend. The high abundance of *Proteobacteria* in the

11-day experiment is in agreement with observations in a study with preterm infants who were fed formula supplemented with HMO from pooled human milk (no specification of HMO were given) (41). The authors report low levels of *Bifidobacteria* and no *Lactobacilli*, concomitantly with increasing numbers of *Clostridia* and an unexpected trend toward an increase in *Proteobacteria* in both groups. Although the interpretation of the data from our piglets is difficult due to the large individual variation, the presence of *Bifidobacteria*, although low in total numbers, was correlated with total HMO contents, and specifically with 2′FL levels in colonic content. The importance of 2′FL particularly for *Bifidobacteria* through the interaction with a newly identified ABC transporter as a key genetic factor for the utilization of 2′FL and other fucosylated oligosaccharides has been thoroughly discussed by Matsuki et al. (42). This could be important, as recent reports indicated a strong association of the secretor genotype with the composition of *Bifidobacteria* in the human intestine (43, 44).

In conclusion, we found that (i) all pigs receiving HMO containing diets excreted these HMO via the colon and urine, but individual variations were large. This data resemble the situation in human infants with no clear excretion pattern neither in term nor in preterm infants; (ii) HMO supplemenation was not related to NEC, bacterial colonization or intestinal permeability; (iii) the 5-day supplementation of the complex 25-HMO blend led to low concentrations of 3-FL and LNFP I in colonic contents, indicating a preferred utilization of these two HMO; (iv) In colon, only the abundance of *Bifidobacteria*, although low in total numbers compared to other microorganisms, correlated with total HMO in colon content and specifically with 2′FL, (v) Increasing the HMO supplementation period to 11 days lowered the fecal excretion of HMO. Thus, intestinal immaturity, together with delayed bacterial colonization and low bacterial diversity, may lead to a different metabolic fate of HMO during the first 1–2 weeks after preterm birth. A sufficient gastrointestinal maturation may be required to observe clear benefits of HMO, both locally in the gut and beyond.

AUTHOR CONTRIBUTIONS

The authors' responsibilities were as follows: SR, NS, PS, and SB designed research; SR, SO, and MR conducted research; SR, SK, and MR analyzed the data; SR, SK, PS, and SB wrote the paper; SR, SO, SK, CK, PS, and SB revised the manuscript. SR, PS, and SB had primary responsibility for the final content. All authors read and approved the final manuscript.

FUNDING

The study was supported by the Innovation Fund Denmark (NEOMUNE grant number 0603-00774B) and Glycom A/S (Lynby, Denmark). The funder Glycom A/S (Lynby, Denmark) provided the HMO blends for the study, but played no role in the study design, the collection, analysis or interpretation of data, the writing of this paper or the decision to submit it for publication.

ACKNOWLEDGMENTS

We thank Dr. Christian Borsch, Cordula Becker, and Christina Meyer from the University of Giessen for their great technical assistance.

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Conflict of Interest Statement: NS is employed by Nestec S.A. at the Nestlé Research Center.

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.

frontiers in Microbiology

MINI REVIEW
published: 25 February 2019
doi: 10.3389/fmicb.2019.00343

Shaping the Infant Microbiome With Non-digestible Carbohydrates

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Natural polysaccharides with health benefits are characterized by a large structural diversity and differ in building blocks, linkages, and lengths. They contribute to human health by functioning as anti-adhesives preventing pathogen adhesion, stimulate immune maturation and gut barrier function, and serve as fermentable substrates for gut bacteria. Examples of such beneficial carbohydrates include the human milk oligosaccharides (HMOs). Also, specific non-digestible carbohydrates (NDCs), such as galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) are being produced with this purpose in mind, and are currently added to infant formula to stimulate the healthy development of the newborn. They mimic some functions of HMO, but not all. Therefore, many research efforts focus on identification and production of novel types of NDCs. In this review, we give an overview of the few NDCs currently available [GOS, FOS, polydextrose (PDX)], and outline the potential of alternative oligosaccharides, such as pectins, (arabino)xylo-oligosaccharides, and microbial exopolysaccharides (EPS). Moreover, state-of-the-art techniques to generate novel types of dietary glycans, including sialylated GOS (Sia-GOS) and galactosylated chitin, are presented as a way to obtain novel prebiotic NDCs that help shaping the infant microbiome.

Keywords: infant, microbiome, non-digestible carbohydrates, exopolysaccharides, transglycosylation

OPEN ACCESS

Edited by:
F. Javier Moreno, Instituto de Investigación en Ciencias de la Alimentación (CIAL), Spain

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Specialty section:
This article was submitted to Systems Microbiology, a section of the journal Frontiers in Microbiology

Received: *23 November 2018*
Accepted: *08 February 2019*
Published: *25 February 2019*

Citation:
Verkhnyatskaya S, Ferrari M, de Vos P and Walvoort MTC (2019) Shaping the Infant Microbiome With Non-digestible Carbohydrates. Front. Microbiol. 10:343. doi: 10.3389/fmicb.2019.00343

INTRODUCTION

Humans live in symbiosis with trillions of bacteria, and most of them are symbionts and beneficial to the host (Sender et al., 2016). Disturbance in our microbiota can contribute to the development of many diseases (Wang et al., 2017). Bacteria are mainly present in the areas that are more exposed to the surrounding environment such as the skin, vaginal and oral mucosa, and the GIT. The gut microbiota has been extensively studied due to its impact on the establishment of immunity (Martin et al., 2010) and prevention of chronic inflammation (Belkaid and Hand, 2014). While the fetal GIT was considered sterile for many years, emerging evidence suggests that colonization of the GIT starts already at the prenatal stage with neonatal colonization by *Enterobacter*, *Escherichia*, *Shigella*, and *Staphylococcus* species, as detected in the umbilical cord, placenta, and amniotic fluid (Carmen Collado et al., 2016). After birth, the newborn gut is rapidly colonized by different bacterial

Abbreviations: APS, acidic polysaccharides; AXOS, (arabino-)xylo-oligosaccharides; DP, degree of polymerization; EPS, exopolysaccharides; FOS, fructo-oligosaccharides; GHs, glycosyl hydrolases; GIT, gastrointestinal tract; GMP, glycomacropeptide; GOS, galacto-oligosaccharides; GTs, glycosyl transferases; H-APS, high-molecular weight acidic polysaccharides; HePS, heteropolysaccharides; HMOs, human milk oligosaccharides; HoPS, homopolysaccharides; NDCs, non-digestible carbohydrates; NEC, necrotizing enterocolitis; NPS, neutral polysaccharides; PDX, polydextrose; POS, pectin oligosaccharides; SB-POS, sugar beet pulp pectin oligosaccharides; SCFAs, short chain fatty acids.

strains with the first colonizers being facultative aerobes such as *Escherichia* and *Enterococcus*, whose oxygen consumption allows colonization of anaerobic bacteria, with the most abundant being *Bifidobacterium* (Houghteling and Walker, 2015). Many early-life factors have an impact on the composition of the infant gut microbiota, including the mode of delivery, the infant feeding pattern, diet composition, and the use of antibiotics, but also the health of the mother during pregnancy (Gonzalez-Perez et al., 2016).

The early colonization process is crucial for a healthy microbiome and prevents disease later in life. Gut microbiota are essential for digestion of food, but also to function as a barrier against pathogens, and for the development of immune tolerance to innocuous antigens and microorganisms (Yang et al., 2016). Imbalances in the intestinal microbiome composition can result in bacterial overgrowth or lower species diversity, making the host more susceptible to pathogenic infections (Lozupone et al., 2012). Furthermore, microbial dysbiosis may lead to autoimmune and allergic diseases. The healthy infant intestinal microbiome has a low microbial diversity, with *Bifidobacterium*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* being most abundant. Feeding has a major influence on the microbiota composition, as breast-fed infants have higher *Bifidobacterium* and *Enterobacteria* numbers and a lower diversity in comparison to formula-fed infants (Milani et al., 2017).

There is a growing understanding of the mechanisms by which a balanced microbiome contributes to health. For instance, many genera such as *Eubacterium* and *Bacteroides* are involved in the production of vitamin K (Rossi et al., 2011), an essential cofactor promoting the γ-carboxylation of glutamate residues involved in blood clotting (Gröber et al., 2014). *Bifidobacterium* species are able to produce folate, a vitamin involved in DNA synthesis and repair with an undisputed importance in neurological development (Crider et al., 2012), with the best producing strains being *Bifidobacterium adolescentis* and *Bifidobacterium pseudocatenulatum* (Rossi et al., 2011). Lactobacilli carry the *rib* operon, which is implicated in the *de novo* synthesis of riboflavin, which is important in developmental processes and in the hemopoietic system (Thakur et al., 2016). Moreover, gut microbiota are responsible for the production of SCFAs, such as acetate, propionate, and butyrate. Acetate is the most abundant, and it is used by many gut commensals to produce propionate and butyrate in a growth-promoting cross-feeding process. SCFAs are important for the reduction of the intestinal pH and the consequent inhibition of pathogen's adhesion. Moreover, butyrate is the preferred energy source for colon epithelial cells, where it contributes to the maintenance of the gut intestinal barrier, exerts immunomodulatory and anti-inflammatory effects (Stilling et al., 2016; Zhang et al., 2018), also through epigenetic mechanisms (Furusawa et al., 2013; Paparo et al., 2014), and may even prevent colorectal cancer (Wu et al., 2018).

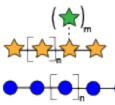
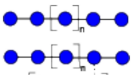
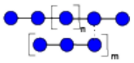
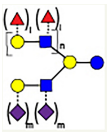
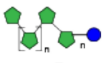
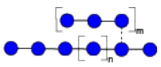
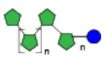
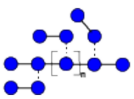
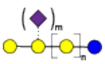
A healthy infant microbiome is normally created under the guidance of molecules in human milk. This is mainly accomplished by HMOs, which serve as feed for specific bacterial species. HMOs are a family of >200 structurally different molecules that vary in quantity and composition from mother to mother, and over the course of lactation. However, some general trends in HMO composition are present (**Table 1**). HMOs are composed of a linear or branched backbone containing galactose (Gal), *N*-acetylglucosamine (GlcNAc), and glucose (Glc), which can be decorated with fucose (Fuc) and sialic acid (Sia) residues, and this decoration pattern depends on the mother's secretory status (Bode, 2012). Only members of *Bifidobacterium* and *Bacteroides* were shown to metabolize HMOs (Marcobal et al., 2010). Especially *Bifidobacterium bifidum* and *Bifidobacterium infantis* are efficient utilizers of HMOs, whereas they are moderately digested by *Bifidobacterium breve* and *Bifidobacterium longum*. Interestingly, *Bifidobacterium animalis* and *B. adolescentis* are incapable of degrading HMOs (LoCascio et al., 2009; Sela and Mills, 2010). To ensure a high number in the gut, bifidobacteria have been observed to create a cross-feeding niche, as the extracellular fermentation of HMOs by *B. bifidum* is associated with a cooperative effect for *B. infantis*, which is able to import the released sugars and digest them intracellularly (Garrido et al., 2012; Thomson et al., 2018).

For infants where human milk is not an option, infant formula supplemented with NDCs that should mimic prebiotic functions of HMOs have been created (Vandenplas et al., 2015). A prebiotic is defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson et al., 2017). HMOs fulfill these criteria, as they are not digested in the upper part of the GIT of infants (Engfer et al., 2000), while they serve as preferred food source for beneficial bacteria. Next to HMOs, other NDCs or dietary fibers have been shown to be major drivers of gut microbiome composition and function, and might be added to infant formula for this purpose (Benitez-Paez et al., 2016). Interestingly, the currently applied molecules do not mimic all the functions of the >200 HMOs found in human milk, so novel oligosaccharides are needed to fill this void. This review aims to inspire the selection of future NDCs that can be added to infant formula by reviewing beneficial glycans that show great promise as modulators of the microbiome, with a focus on their interaction with bifidobacteria and lactobacilli, since most is known about these genera. Moreover, state-of-the-art techniques to generate novel types of dietary glycans are presented.

NDCS CURRENTLY ADDED TO INFANT FORMULA

To mimic the beneficial effects of HMOs, two alternative oligosaccharides are routinely added to infant formula: GOS and FOS (**Table 1**). GOS are produced by enzymatic transglycosylation from lactose (vide infra), providing a mixture of differently linked oligosaccharides with a DP from 2 to 8. The Gal units are linked through β-galactosidic linkages, which are resistant to GIT enzymes until they reach the colon where they are fermented by bacteria. In general, GOS stimulate the growth of bifidobacteria (Absmanner et al., 2010), and especially the numbers of *B. adolescentis* are impacted (Sierra et al., 2015). FOS are generally produced by enzymatic digestion from naturally isolated inulin, yielding oligosaccharides with DP from 2 to 9, and bifidobacteria readily grow when FOS are used as a sole carbon source (Macfarlane et al., 2008).

TABLE 1 | Overview of oligosaccharide structures discussed herein.

Oligosaccharide	Structure	Length	Average structure
Naturally isolated			
Arabinoxylan oligosaccharides (AXOS)	$(\alpha\text{-}1,2/1,3\text{-}Ara)_m\text{-}\beta\text{-}1,4\text{-}Xyl_n$	DP ~ 5	
Curdlan	$(\beta\text{-}1,3\text{-}Glc)_n$	60–2000 kDa	
Dextran	$\alpha\text{-}1,6\text{-}Glc_n$, with *m* branches at α-1,2/1,3-Glc	40–2000 kDa	
Human milk oligosaccharides (HMOs)	$(\alpha\text{-}Fuc)_l/(\alpha\text{-}Sia)_m\text{-}(\beta\text{-}Gal\text{-}\beta\text{-}1,3/1,4\text{-}GlcNAc)_n\text{-}\beta\text{-}Glc$	DP 3–25	
Inulin	$(\beta\text{-}2,1\text{-}Fru)_n\text{-}\beta\text{-}Glc$	DP 10–26 (Raftiline)	
Laminarin	$\beta\text{-}1,3\text{-}Glc_n$, with *m* branches at β-1,6-Glc	DP 20–30	
Levan	$(\beta\text{-}2,6\text{-}Fru)_n$	~500 kDa	
Pectin	$(\beta\text{-}1,4\text{-}Gal)_k/(\alpha\text{-}1,5\text{-}Ara)_l\text{-}(\alpha\text{-}1,4\text{-}GalA\text{-}\alpha\text{-}Rha)_m\text{-}(\alpha\text{-}1,4\text{-}GalA)_n$	N/A	
Xylo-oligosaccharides (XOS)	$(\beta\text{-}1,4\text{-}Xyl)_n$	DP 2–10	
Enzymatically produced			
Fructo-oligosaccharides (FOS)	$(\beta\text{-}2,1\text{-}Fru)_n\text{-}\beta\text{-}Glc$	DP 2–9	
Galacto-oligosaccharides (GOS)	$(\beta\text{-}1,3/1,4/1,6\text{-}Gal)_n\text{-}\beta\text{-}Glc$	DP 2–8	
Gal-chitin	$(\beta\text{-}1,4\text{-}Gal)_m\text{-}\beta\text{-}1,4\text{-}GlcNAc_n$	DP 2–4	
Gal-chitosan	$(\beta\text{-}1,4\text{-}Gal)_m\text{-}\beta\text{-}1,4\text{-}GlcN_n$	DP 2–4	
Polydextrose (PDX)	$(\alpha/\beta\text{-}1,2/1,3/1,4/1,6\text{-}Glc)_n$	DP 5–25	
Sia-GOS	$(\alpha\text{-}2,3\text{-}Sia)_m\text{-}(\beta\text{-}1,3/1,4/1,6\text{-}Gal)_n\text{-}\beta\text{-}Glc$	DP 2–8	

Nomenclature: arabinose (Ara, ★); fructose (Fru, ⬟); fucose (Fuc, ▲); galactose (Gal, ●); galacturonic acid (GalA, ◇); glucosamine (GlcN, ◪); N-acetylglucosamine (GlcNAc, ■); glucose (Glc, ●); rhamnose (Rha, ▲); sialic acid (Sia, ◆); xylose (Xyl, ★).

When mixtures of GOS/FOS in a 9/1 ratio are used, the ratio of different *Bifidobacterium* species was similar to breast-fed infants (Haarman and Knol, 2005). This GOS/FOS mixture was also demonstrated to be the best growth substrate for *Bifidobacteria* and *Lactobacilli*, while inulin and PDX led to poor growth (Vernazza et al., 2006). PDX is a synthetic polymer of randomly connected Glc units with an average DP of 12 and all possible glucosidic linkages: α- or β- and 1→2, 1→3, 1→4, and predominantly 1→6 (Ramiro do Carmo et al., 2016). When PDX was used in combination with GOS in a 1:1 ratio, the increase in *Bifidobacterium* species, specifically *B. infantis*, *B. longum*, and *B. catenulatum*, was similar to the breast-fed microbiota, where *B. infantis*, *B. longum*, and *B. breve* are predominant (Scalabrin et al., 2012). Interestingly, this GOS/PDX mixture was also identified in a commercial brand of infant formula (Nijman et al., 2018). Next to prebiotic properties, GOS, FOS, and mixtures of both components were also shown to have immunomodulatory properties, which have recently been reviewed (Macfarlane et al., 2008; Ackerman et al., 2017, Akkerman et al., 2018).

ALTERNATIVE NDCS ISOLATED FROM NATURAL SOURCES

Polysaccharides with prebiotic potential have mostly been extracted from the cell wall of higher plants including cereals and grains, fruits, and vegetables, seaweeds, and microalgae (de Jesus Raposo et al., 2016). In this section, we focus on the naturally isolated polysaccharides POS and AXOS that have

already been investigated for their prebiotic effect and might serve as alternative for HMOs.

Pectins have received widespread attention for their potential as prebiotics. They are composed of a backbone of galacturonic acids, which are hypothesized to mimic the Sia residues in HMOs (**Table 1**). Pectins are heteropolysaccharides and are available from citrus peels, apple pomace, sugar beet pulp, and potato pulp. The hydrolysis of pectins yields POS, which are composed of galacturonic acid, galactose, rhamnose, arabinose, and xylose building blocks. Moreover, POS can be methylated or esterified on the galacturonic acid residues, and the degree of methylation, esterification, and the ratios of monosaccharides depends on the source of pectin and the type of extraction method used. In light of this structural diversity, studies with POS become more reliable and reproducible when the exact molecular structure is described. POS has a demonstrated prebiotic effect, promoting the growth of *Bifidobacteria* and *Lactobacilli*. Interestingly, especially neutral POS, such as galactan, GOS, arabinan, and arabino-oligosaccharides, enhance the growth of *Bifidobacteria* to a similar extent as inulin (Onumpai et al., 2011; Di et al., 2017). A similar increase in bifidobacteria numbers was observed for an arabinose-rich mixture of SB-POS, while lactobacilli were selectively enhanced using lemon peel waste-derived POS, which was high in galacturonic acids, and the number of bacterial members of *Faecalibacterium prausnitzii* group and *Roseburia intestinalis* (both of the phylum Firmicutes) increased with all types of pectins (Gomez et al., 2016). In contrast, a commercial source of SB-POS, which was shown to contain a high galacturonic acid content, had little effect on numbers of bifidobacteria, highlighting the importance of the pectin composition (Leijdekkers et al., 2014). Infant formula with pectins has been studied in human infant trials, but there was no effect of the acidic oligosaccharides on bifidobacteria and lactobacilli (Fanaro et al., 2005).

Xylo-oligosaccharides (XOS, **Table 1**) are present in fruits, vegetables, bamboo, honey, and milk, and can be produced on an industrial scale by enzymatic degradation of xylan-rich materials (Aachary and Prapulla, 2011). XOS is readily fermented by commensal bacteria, and can in humans increase the population of fecal bifidobacteria and SCFA production (Lecerf et al., 2012). AXOS (**Table 1**) are prepared by degradation of arabinoxylan, which is the major non-cellulose polysaccharide in cereals and plants. In a fermentation study, it was shown that *B. longum* B24 could liberate the arabinose units from AXOS without degrading the xylan backbone, while *B. longum* B18 was able to metabolize XOS up to DP4 (Riviere et al., 2018). *B. adolescentis* B72 degraded various types of FOS, partially degraded inulin, and metabolized XOS longer than DP4. The authors suggested that the strain-specific mechanisms to utilize different glycans lead to a cooperative effect and simultaneous striving of different bacterial strains. A similar cross-feeding effect was observed between *B. longum* NCC2705 and *Eubacterium rectale* ATCC 33656 when grown on AXOS (Riviere et al., 2015). *B. longum* is able to release arabinose and produce acetate, whereas *E. rectale* uses acetate to produce butyrate. When co-cultured on AXOS, the consumption of arabinose by *B. longum* and concomitant release of acetate allowed *E. rectale* to produce butyrate, resulting in a simultaneous prebiotic and butyrogenic effect (Riviere et al., 2016). Other examples of such a commensal cross-feeding relationship with bifidobacteria have been reported, including *Faecalibacterium* (De Vuyst and Leroy, 2011; Moens et al., 2016). Negatively charged XOS structures, containing glucuronic acid units, have also been isolated from hardwood (Rivas et al., 2017), and may be promising candidates for novel charged prebiotic NDCs (vide infra).

POTENTIAL OF EXOPOLYSACCHARIDES AS NOVEL NDCS

Exopolysaccharides produced by Gram-positive bacteria currently attract a great deal of attention because of their wide range of beneficial properties (Ryan et al., 2015). Regularly new EPS structures are identified that have a specific health effect, and especially the immune-modulating properties are often investigated (Castro-Bravo et al., 2018). From recent reviews on the characterized EPS structures of *Lactobacillus* and *Bifidobacterium*, their great structural diversity is immediately apparent (Hidalgo-Cantabrana et al., 2014; Castro-Bravo et al., 2018; Oleksy and Klewicka, 2018). They are broadly divided into HoPS, which are composed of a single sugar building block, and HePS, which display a repeating fragment of two to eight different sugar units.

Most HoPS are found to be susceptible to fermentation by commensal bacteria (Salazar et al., 2016), which is presumably directly linked to their relatively simple molecular structure, albeit that they can be very large in size. For instance, the prebiotic effect of β-fructans was investigated with two levan-type EPS isolated from *Lactobacillus sanfranciscensis*, and compared with levan (fructan with β-2,6 linkages, **Table 1**), inulin (fructan with β-2,1 linkages), and FOS (Dal Bello et al., 2001). An enrichment of *Bifidobacterium* species in human fecal samples in a large bowel model medium was observed with the EPS and inulin as added carbon source, while levan and FOS had no effect. This may reflect the importance of both the length of the carbohydrate, and the fructose linkage type in the isolated EPS, which may be different from commercial levan. The capability of *Bifidobacterium* species to directly metabolize the *L. sanfranciscensis* EPS was further demonstrated in a fermentation study (Korakli et al., 2002). β-Glucans, including curdlan (linear β-1,3-Glc, **Table 1**) and laminarin (β-1,3/1,6-Glc, **Table 1**), are also readily fermented by bifidobacteria. Especially the *B. infantis* population benefitted from β-glucan digestion, and concomitant increased production of propionate and butyrate was observed (Zhao and Cheung, 2011).

In contrast, there is a lack of data on the digestibility of HePS by commensal bacteria, presumably due to their complex structures and generally low isolated yields. Both bifidobacteria and lactobacilli display structurally diverse HePS, which may contain galacto-pyranose and -furanose, rhamnose, mannose, and 6-deoxy-talose, among others

(Hidalgo-Cantabrana et al., 2014). In a fecal slurry fermentation experiment, the uncharacterized EPS from different *B. animalis*, *B. pseudocatenulatum*, and *B. longum* species isolated from humans were investigated for their prebiotic effect (Salazar et al., 2008). Although there were high inter-individual variations, the data indicated an EPS-related enrichment of *Bifidobacterium* species, similar to the result obtained with inulin. *Bacteroides fragilis* DSMZ 2151 was also found to digest (uncharacterized) HePS from *B. longum* E44 and *B. animalis* subsp. *lactis* R1, with concomitant increase in propionate and acetate production (Rios-Covian et al., 2016). Although there is no data on fermentation yet, an interesting link between acidic phosphate groups in HePS structures and immune responses was found (Kitazawa et al., 1998). *Lactobacillus delbrueckii* subsp. *bulgaricus* OLL-1073-R1 produces two different EPS: acidic phosphate-containing (APS) and NPS, both composed of Glc and Gal residues (ratio 3:2). Interestingly, only the APS was a strong inducer of proliferation and activity of macrophages. When the APS was fractionated in two different EPS based on size, the B-cell mitogenic activity was observed only with high-molecular weight polysaccharide (H-APS). The impact of the acidic phosphate was substantiated by chemical dephosphorylation, which resulted in a reduction of the stimulatory effect (Kitazawa et al., 1998). Interestingly, when unrelated dextran (α-Glc HoPS from *Leuconostoc mesenteroides*, **Table 1**) was chemically phosphorylated, the proliferation of lymphocytes was directly proportional to the phosphate content (Sato et al., 2004). Unfortunately, there is no information available on the fermentability of these charged EPS, which could shed a light on their prebiotic potential. Overall, the structural complexity of especially the HePS yields large promise for prebiotic potential, which warrants extra dedication to unraveling the molecular structure of prebiotic HePS to gain more insight in the structure–function relation.

DEVELOPMENT OF NOVEL NDCS

With the increasing interest and appreciation of the impact of dietary glycans on healthy microbiome development and overall human health, there is a tremendous surge in methods to produce existing and novel glycans. Chemical synthesis has the potential to generate well-defined carbohydrate structures, but reliable methods are not generally available, and especially not on the scale that would allow for biological evaluation. Enzymatic synthesis is more amenable to larger scale carbohydrate production, but also has its challenges. GTs have successfully been used in the synthesis of HMO structures *in vitro* (Chen et al., 2015; Yu et al., 2017a), but their application is hampered by the use of expensive nucleotide-activated sugars, and multi-enzyme substrate recycling systems are needed to prevent metabolites from inhibiting enzyme activity (Qin et al., 2016). Using bacterial cells as production factories however, major advancements in HMO production have been made and have resulted in FDA approval and commercialization of the major HMO 2′-fucosyllactose. Different methods are now available in *Saccharomyces cerevisiae* (Yu et al., 2018) and *Escherichia coli* (Chin et al., 2017), and other HMO structures are expected to be produced in this way in the near future (Sprenger et al., 2017). Alternative methods rely on the use of GHs, which are able to perform a transglycosylation reaction next to glycosidic bond hydrolysis (Danby and Withers, 2016; Manas et al., 2018). In this way, well-known prebiotic fibers such as GOS are industrially produced by making use of β-galactosidase enzymes (Torres et al., 2010), and also FOS can be synthesized in this way (Karboune et al., 2018). This approach can also be used to decorate existing glycans with other sugars, and the generation of galactosylated, fucosylated (Zeuner et al., 2018), and sialylated glycans as HMO mimics have recently been reviewed (Zeuner et al., 2014). A variety of glycan acceptors, ranging from monosaccharides and lactose to Tn antigens (e.g., *N*-acetylgalactosamine-threonine conjugates), GOS, and HMOs have been described. This strategy has the potential to rapidly yield novel dietary glycans that display complex sugar building blocks (e.g., Sia, Fuc) that were previously difficult to obtain.

A successful example of this strategy is the production and biological evaluation of sialylated GOS (Sia-GOS, **Table 1**). Using a transsialidase from *Trypanosoma cruzi* and bovine κ-casein-derived GMP as the source of Sia, commercial GOS was decorated with α-2,3-Sia residues to create mono-Sia-GOS (Wilbrink et al., 2015). These novel glycans were subsequently tested in a rat model of NEC, an intestinal disorder mainly observed in preterm infants, for which sialylated HMOs were found to protect (Jantscher-Krenn et al., 2012; Yu et al., 2017b). Interestingly, Sia-GOS significantly reduced the pathology score of NEC, with pooled HMO still being superior in terms of protection, while regular GOS supplementation and formula-feeding both resulted in the worst pathology scores (Autran et al., 2016). In separate fermentation studies, with a Sia-GOS batch produced by a GT-catalyzed sialylation, it was revealed that *B. infantis* ATCC 15697 was able to digest Sia-GOS, whereas *B. adolescentis* ATCC 15703 could not, highlighting the species-specific ability to metabolize HMOs and HMO mimics (Wang et al., 2015).

Using a similar strategy, chitin and chitosan (deacetylated at the amine) oligosaccharides were decorated with β-Gal residues (Black et al., 2014). The transglycosylation was performed with β-galactosidase from *Lactobacillus plantarum* with lactose as the Gal source, and different chitin and chitosan acceptors were decorated with one to three residues in a β-1,4 linkage (**Table 1**). Especially the β-Gal-chitosan and GOS oligosaccharides were found to prevent enterotoxigenic *E. coli* K88 from adhering to porcine erythrocytes, in contrast to alpha-linked GOS and α-Gal-chitosan (Yan et al., 2017; Yan and Ganzle, 2018). It will be interesting to perform digestion studies of these novel β-Gal-chitosan glycans by bacteria to investigate their prebiotic effect.

CONCLUDING REMARKS

It is clear that the creation of a healthy infant microbiome is a delicate interplay of a variety of commensal bacteria, which

can be beneficially influenced by oligosaccharides. Because the composition of the infant's microbiome can have a profound effect on adult life, there is a great potential for the addition of carbohydrates that mimic HMO functions. Promising better candidates that may substitute or be added to currently applied NDCs are the HePS, which have the potential to specifically enhance certain species. Also, as structural mimics of HMOs, fucosylated and sialylated oligosaccharides are expected to be applied in the near future. In the end, more knowledge of the presence of the biosynthetic machinery necessary to utilize specific oligosaccharides will pave the way for the development of novel NDCs with prebiotic effects.

AUTHOR CONTRIBUTIONS

SV, MF, and MW contributed to the organization and structure of the review. All authors contributed to the writing and critical evaluation of the final version.

FUNDING

Within the framework of the Carbohydrate Competence Center, this research has been financially supported by the Netherlands Organization for Scientific Research (NWO). MW acknowledges financial support from the European Union through the Rosalind Franklin Fellowship COFUND project 600211.

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Conflict of Interest Statement: The 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.

frontiers in Nutrition

MINI REVIEW
published: 24 April 2019
doi: 10.3389/fnut.2019.00050

Structures and Metabolic Properties of Bovine Milk Oligosaccharides and Their Potential in the Development of Novel Therapeutics

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Among the many bioactive components in human milk, the free oligosaccharides (OS) have been intensely studied in recent decades due to their unique ability to selectively modulate the infant gut microbiota, in addition to providing numerous other health benefits. In light of the demonstrated value of these compounds, recent studies have set out to characterize the structures and properties of the similar and more widely-available OS in the dairy industry. This mini review gives a brief overview of the common analytical techniques used to characterize bovine milk OS and highlights several recent, key studies that have identified valuable physiological and metabolic effects of these molecules *in vivo*. Although traditionally considered indigestible by human enzymes, evidence now suggests that milk OS are partially absorbed in the intestines and likely contribute to the development of molecular structures in the brain. Furthermore, aside from their prebiotic effects, these compounds show promise as therapeutics that could alleviate numerous metabolic abnormalities, including undernutrition, obesity, and excessive intestinal permeability. The need for novel treatments to address these and related health issues is motivating the development of scalable techniques to produce large quantities of milk OS for use as food ingredients. The safety and tolerability of high dosages of bovine milk OS have been demonstrated in two independent human studies, which potentially opens the door for further research aiming to utilize these molecules to alleviate common metabolic health issues.

Keywords: milk, prebiotic, obesity, mass spectrometry, therapeutic

OPEN ACCESS

Edited by:
Oswaldo Hernandez-Hernandez, Instituto de Investigación en Ciencias de la Alimentación (CIAL), Spain

Reviewed by:
Vasiliki Kachrimanidou, University of Reading, United Kingdom
Douwe Van Sinderen, University College Cork, Ireland

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Specialty section:
This article was submitted to Nutrition and Food Science Technology, a section of the journal Frontiers in Nutrition

Received: *27 February 2019*
Accepted: *04 April 2019*
Published: *24 April 2019*

Citation:
Robinson RC (2019) Structures and Metabolic Properties of Bovine Milk Oligosaccharides and Their Potential in the Development of Novel Therapeutics. Front. Nutr. 6:50. doi: 10.3389/fnut.2019.00050

INTRODUCTION

Milk harbors a suite of bioactive compounds, including free oligosaccharide (OS) structures that are well-characterized as selective prebiotics and that play an important role in infant health and development (1, 2). Research in the last few decades has made immense progress in characterizing the beneficial biological functions of OS and uncovering the mechanistic pathways by which they are exerted. The majority of studies on milk OS functionality initially focused on the highly-concentrated OS in human milk. Human milk OS have been extensively profiled, with as many as 200 structures being identified in comprehensive studies (3, 4), and the ability of gut-associated bacteria to consume many of these structures is well-documented (5–7). Several gut bacterial

species, including select species of *Lactobacillus* and *Bifidobacteria,* are highly desirable due to their ability to down-regulate an over-active immune system and reduce inflammatory response (8). This research field has now expanded to identify the similar structures and bioactivities of milk OS from other mammalian species. In particular, industrial production of bovine milk has prompted studies into the therapeutic value of bovine milk oligosaccharides (BMOs) and the dairy industry's relatively underutilized BMO-containing side streams. This mini-review highlights recent studies that demonstrate novel bioactivities of BMOs, with a particular focus on their digestibility and metabolic effects. The paper also provides an overview of the current analytical tools used in OS characterization and the development of industrial-scale processes for BMO production. Considering the wide availability of BMOs in dairy streams and the current need for therapeutics with BMO-like functionalities, these molecules show promise as a solution to epidemic metabolic and digestive illnesses.

BOVINE MILK OLIGOSACCHARIDE COMPOSITION AND STRUCTURES

Oligosaccharides in bovine milk are assembled in the mammary gland by combining the monosaccharides glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc), N-acetylgalactosamine, fucose, and the sialic acids N-acetylneuraminic acid and N-glycolylneuraminic acid (9, 10). The OS structures contain either lactose (Gal(β1-4)Glc) or N-acetyllactosamine (Gal(β1-4)GlcNAc) at their reducing end, with additional monosaccharide residues branching off from the non-reducing galactose (9, 10). In some cases, the BMOs possess lacto-N-biose (Gal(β1-3)GlcNAc) or N-acetyllactosamine units linked to the lactose core, which are defining features of the type 1 and type 2 OS structures contained within many human milk OS (**Figure 1C**) (1, 9). The collection of BMOs found in milk and colostrum has been extensively profiled by our research group, with 30–50 structures typically being identified in comprehensive studies (9, 12–14). Although bovine milk contains fewer OS structures than human milk, the two share at least 10 common structures (**Figure 1C**), including the acidic 3′-sialyllactose and 6′-sialyllactose. These two OS comprise a large percentage of the BMO pool (9, 11). Complete or partial structures are known for many BMOs (9); however, there remains a large proportion of BMOs for which only monosaccharide compositions are known. For example, recent studies have identified several large fucosylated OS in bovine milk, but to date only the monosaccharide compositions have been determined (9, 15, 16). A more complete structural characterization of the entire BMO pool could improve our understanding of their bioactivities and digestibilities by microbes. In many cases, linkage types influence functionality, as exemplified by *in vivo* studies showing the ability of α1-2–linked fucosyloligosaccharides to prevent *Campylobacter jejuni* infection (17).

Abbreviations: BMO, bovine milk oligosaccharide; Gal, galactose; Glc, glucose; GlcNAc, N-acetylglucosamine; LC-MS, liquid chromatography—mass spectrometry; OS, oligosaccharide.

ANALYTICAL METHODOLOGIES FOR MILK OS

Identification of the individual OS structures in milk has come with numerous analytical challenges, many of which have been resolved in the past two decades. The free OS in milk are frequently analyzed by liquid chromatography-mass spectrometry (LC-MS, **Figure 1A**) (12, 16, 18). This technique is utilized for initial discovery and profiling of the entire collection of OS in a sample. It conveniently provides relative compound abundances and monosaccharide compositions for a multitude of OS within a single experiment. Common LC-MS strategies for OS analysis have been recently reviewed in greater detail elsewhere (19). However, the potential for branching within an OS structure, as well as the numerous possible linkages between neighboring monosaccharides, often requires further experiments to achieve complete structural elucidation.

In some cases complementary techniques are used along with MS for in-depth characterization of OS structures. For example, Aldredge et al. (9) fractionated the bovine milk OS pool by high performance liquid chromatography, incubated each bovine milk OS with glycosidases of known specificity, and analyzed the changes produced with LC-MS. This labor-intensive approach determined a variety of glycosidic linkages and specific monosaccharide types for numerous OS (9). A similar approach was used by Wu et al. in the determination of human milk OS structures (3, 20). Alternatively, more rapid approaches that do not rely on pre-fractionation use strategic derivatization and subsequent analysis of the hydrolyzed monosaccharides to provide at least partial linkage information. Galermo et al. have recently published a method by which the monosaccharides and linkage types present in oligo- and polysaccharides can be determined in a high-throughput manner using a pair of derivatization strategies and LC-MS (21).

Not surprisingly, the multitude of potential glycosidic linkages and the efforts required to deduce complete OS structures has hindered chemical synthesis of larger OS structures for use as analytical standards. However, several standards are now available for the smaller bovine milk OS, allowing absolute quantification of a subset of the OS ensemble (18, 22). When analytical standards are unavailable, OS abundances are often measured in relative terms. This can be done using measures such as mass spectral peak height or chromatographic peak area of OS to compare abundances among samples, known as a "label free" relative quantification. Alternatively, several derivatization strategies have been developed that allow relative abundances of isotopically-labeled carbohydrates to be compared on the basis of the intensity of their unique mass spectral peaks. Some examples include reducing-end derivatization of glycan sample pairs with a heavy/light label pair (23, 24) or with a series of isobaric reagents (**Figure 1B**) (25, 26). Further details of these relative quantification techniques and derivatization strategies have been

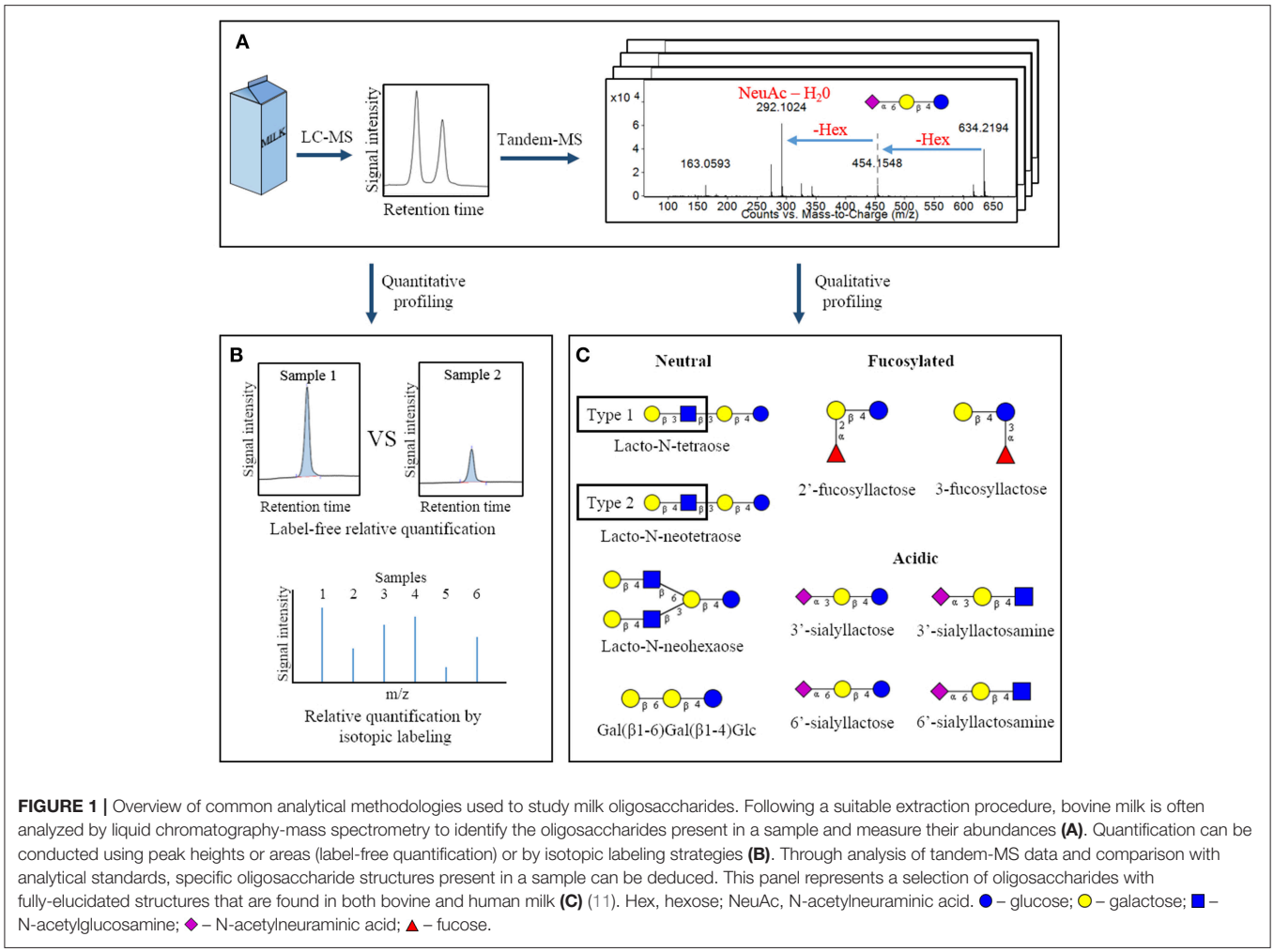

FIGURE 1 | Overview of common analytical methodologies used to study milk oligosaccharides. Following a suitable extraction procedure, bovine milk is often analyzed by liquid chromatography-mass spectrometry to identify the oligosaccharides present in a sample and measure their abundances **(A)**. Quantification can be conducted using peak heights or areas (label-free quantification) or by isotopic labeling strategies **(B)**. Through analysis of tandem-MS data and comparison with analytical standards, specific oligosaccharide structures present in a sample can be deduced. This panel represents a selection of oligosaccharides with fully-elucidated structures that are found in both bovine and human milk **(C)** (11). Hex, hexose; NeuAc, N-acetylneuraminic acid. ● – glucose; ● – galactose; ■ – N-acetylglucosamine; ◆ – N-acetylneuraminic acid; ▲ – fucose.

described in a recent book chapter by Orlando (27) and in a review by Dong et al. (28), Robinson (29).

BOVINE MILK OLIGOSACCHARIDE CONTENT AND KNOWN PHENOTYPIC VARIATIONS

Although analytical standards for quantification do not yet exist for many BMOs, the total OS concentration in bovine milk is estimated at approximately 1–2 g/L in colostrum and 100 mg/L in mature milk (18, 30, 31). Despite the current difficulties in quantifying some OS structures, analytical studies have deduced a wealth of relevant information on OS production in cows. During the first week of lactation, BMO abundances drop relatively quickly and decline somewhat further as cows transition to mature milk (14, 18). Bovine colostrum is a particularly rich source of these OS, and processing streams within the dairy industry have the potential to serve as a raw material for BMO isolation. During cheese production, non-casein proteins and polar molecules such as salts, lactose, and BMOs are eliminated from the cheese as whey. Purification of the whey proteins produces a liquid byproduct known as whey permeate, a stream that contains BMOs (32). Although uses for whey permeate have been identified, it is often considered a waste stream (33), and dried whey permeate and its byproducts are typically sold at low prices (34). Therefore, recovery of BMOs from whey permeate could add value to this stream and improve dairy industry sustainability. Pilot-scale techniques to isolate OS from this dairy stream have been developed using membrane filtration (35, 36). The wide availability of dairy side streams that contain BMOs could allow these processing techniques to feasibly produce isolated milk OS for functional testing and therapeutic applications (**Figure 2**). As described in the following sections, isolated BMOs have demonstrated beneficial health effects in a variety of *in vivo* studies.

Several studies have explored variations in milk OS abundances within dairy cattle in order to characterize industrial BMO availability and to elucidate factors that influence OS production. The Holstein-Friesian and Jersey breeds are commonly used for milk production, and several studies have examined differences in OS production among these and related breeds (14, 16, 30). Most recently, we have profiled

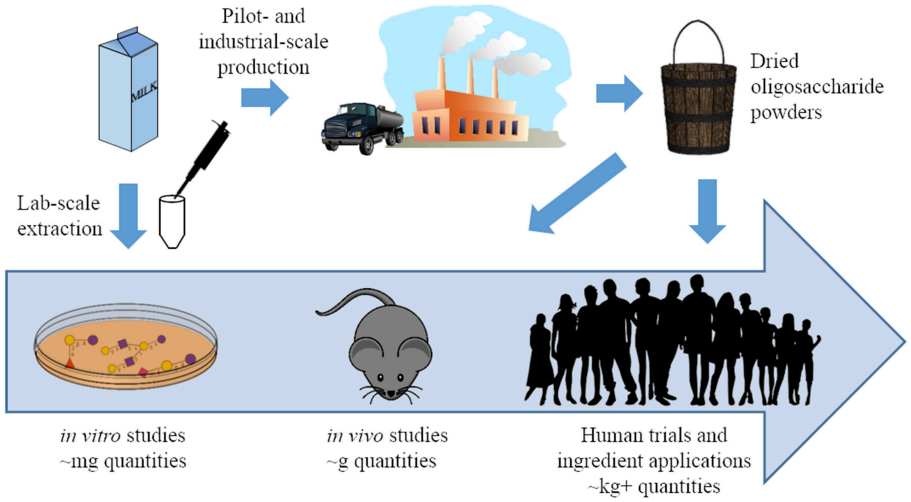

FIGURE 2 | A pictorial representation of the progress made in milk oligosaccharide functional testing and isolation strategies over the past 20 years. Previously, functional testing was generally limited to *in vitro* studies conducted with oligosaccharides isolated from milk at lab scale. Synthetic and pilot-scale isolation can now generate these compounds in larger quantities, and oligosaccharides from both sources have been used in *in vivo* studies and clinical trials (37).

milk OS abundances in a total of 634 samples collected from these breeds and have measured greater amounts of most OS in milk from the Jersey breed (38). The Jersey cows, however, also showed much greater cow-to-cow variability. In light of the fact that environmental sources of variation were controlled, these results may reflect an underlying genetic influence on OS production. A recent study by Liu et al. examined the OS content of genotyped Australian Holstein cows and measured high heritabilities for many OS, indicating that the variation in OS abundances between cows were substantially influenced by genetics (39). The study also identified numerous quantitative trait loci, or regions of the genome which likely influence OS abundances (39). Further studies on this topic will be integral to complete elucidation of the pathways responsible for BMO synthesis. While it is suspected that free milk OS are synthesized by some of the same enzymatic pathways that are used in protein-linked glycan synthesis, investigating the genetic influence on OS production should provide more concrete proof of this possibility. This knowledge could also enable implementation of selective breeding strategies to increase the levels of BMOs in milk without requiring genetically modified organism-based approaches.

DIGESTIBILITY: LOCALIZED AND SYSTEMIC ACTIVITIES

Free milk OS are typically considered indigestible by human enzymes (40–42). Nonetheless, there are several reports of human milk OS existing in infant blood (43) and urine (44–46), indicating that a portion of these molecules are absorbed and circulate in the body. The degree of absorption appears to vary substantially by structure (44), and the biological implications of this absorption have yet to be fully elucidated. It has been hypothesized that absorbed OS can prevent urinary tract infections in infants (47), and recent *in vivo* evidence demonstrates that consumption of 3′-sialyllactose and 6′-sialyllactose increases brain ganglioside-bound sialic acid content in piglets (48). Dietary supplementation with various forms of sialic acid (free or bound to milk OS or protein-linked glycans) has improved learning and increased brain sialic acid content in animal studies (49–51), suggesting that these carbohydrates make an important contribution to brain development.

Milk OS that are not absorbed are available for consumption by the gut microbiota. Human milk has long been known to influence the development of the infant gut microbiota in ways that confer health benefits to the infant, and more recent studies have determined that the milk OS are key to providing this prebiotic functionality (1, 5, 7, 11, 52). These OS selectively feed specific bacterial species that possess the enzymes necessary to metabolize the wide variety of glycosidic linkages found in OS (7, 11, 53). Several of these prebiotic OS from human milk, including lacto-N-tetraose and the sialyllactose isomer pair, are also found in bovine milk (9, 40), and the ability of the BMO ensemble to modulate the gut microbiota *in vivo* has recently been demonstrated (54, 55). Considering the wide availability of dairy side streams from which these OS can be isolated, BMOs show promise as future therapeutics that could be used to provide human milk OS-associated health benefits to infants and adults at a large scale. Initial studies utilizing these OS from dairy streams have revealed a variety of metabolic benefits resulting from BMO consumption, which are reviewed in the following section.

METABOLIC EFFECTS

A well-studied metabolic impact of prebiotic carbohydrates is the ability to indirectly influence short-chain fatty acid (SCFA)

production in the intestine by promoting the growth of SCFA-generating bacteria (56, 57). SCFAs are products of anaerobic bacterial fermentation that occurs in the gastrointestinal tract. The major SCFAs produced by the gut microbiota are acetate, butyrate, and propionate. These bacterial metabolites are used as substrates for a variety of host processes, including cholesterol synthesis and gluconeogenesis in the liver, as well as serving as a key energy source for colonocytes (58). The bacterial genera *Bifidobacterium* and *Bacteroides*, each of which contain well-characterized milk OS consumers (5, 7, 59), are contributors to SCFA production (56, 60). Recently, our research group has shown that BMO supplementation alone significantly increased the expression of butyrate-generating bacterial genes in western diet-fed mouse models (55). Aside from being the preferred energy source for colonocytes (58), butyrate can have anti-inflammatory effects in the liver and colon (61).

Isolation of milk OS from dairy streams has enabled experiments identifying novel metabolic effects of OS *in vivo*. A study by Charbonneau et al. used animal models of infant undernutrition to show that dietary supplementation with BMOs provides a microbially-mediated increase in lean body mass and bone growth, and generates metabolite profiles indicative of improved nutrient utilization (62). These results were characterized in both gnotobiotic mice and piglets, and they provide striking evidence that milk OS, in combination with the gut microbiota, play a substantial role in development and regulation of metabolic pathways. Although other non-milk carbohydrate polymers, such as inulin, share some of the properties of milk OS, this study revealed that the metabolic changes induced by milk OS were not duplicated with inulin supplementation (62). Therefore, the unique functionalities of milk OS may be imparted by their higher diversity of monosaccharide types and linkages compared to the less structurally diverse prebiotic polymers.

The availability of a pilot-scale supply of milk OS has also led to key experiments demonstrating the beneficial effects of OS on the development of obesity and intestinal permeability. With the prevalence of overweight adults reaching nearly 40% worldwide, and obesity at 13% worldwide (63), novel strategies to combat this unfavorable metabolic state could lead to widespread improvements in health status and reduce healthcare costs arising from obesity-associated illnesses. A growing body of evidence is establishing a causal relationship between gut microbial dysbiosis, intestinal permeability, and the onset of obesity. Weight gain from diet-induced obesity occurs simultaneously with altered intestinal permeability and gut microbial profiles, as well as decreases in anti-inflammatory cytokine expression (64, 65). Furthermore, transplantation of the gut microbiota from an obese individual to germ-free mice can induce elevated weight gain in the mice (66, 67), highlighting the gut environment as a potential target for therapeutic interventions. Though not entirely understood, it is possible that obesity onset is at least partially initiated by increases in circulating bacterial endotoxin as a result of altered intestinal permeability, as outlined in a previous review (68). Therefore, treatments that maintain gut barrier function and/or reduce endotoxin circulation could lead to viable interventions to prevent obesity and its related metabolic conditions. Studies investigating the ability of BMOs to modulate intestinal permeability have shown promising results. Hamilton et al. recently showed that consumption of an ensemble of BMOs can significantly reduce weight gain and reduce the intestinal permeability that is induced in mice consuming a high-fat diet (54). Dietary supplementation with BMOs also increased SCFA abundance in the cecum, an effect that was not replicated by inulin supplementation (54). In a similar study, introduction of BMOs to the diet of high fat-fed mice, in combination with a weekly gavage of the probiotic *Bifidobacterium longum* subspecies *infantis,* prevented increases in intestinal permeability otherwise associated with the high-fat diet (69).

The prebiotic effect of milk OS could be another significant factor contributing to obesity prevention. The presence of bifidobacteria in the mouse gastrointestinal tract is correlated with reduced plasma and intestinal endotoxin levels (70, 71). Conversely, the gut microbiota of high fat-fed mice is associated with increased endotoxin levels. Cani et al. have shown that administration of broad-spectrum antibiotics to mice consuming a high fat diet reduces plasma endotoxin levels to that of the control mice, while antibiotic administration to the control mice produced no significant change in plasma endotoxin (65). The presence of bifidobacteria has also been correlated with a reduction in diabetic symptoms, including improved glucose tolerance (70). Modulation of the gut microbiota may therefore be another promising strategy to prevent these prevalent metabolic issues. Furthermore, although prevention of the obese phenotype is a major clinical target, prevention of gut dysbiosis and excessive intestinal permeability will likely have other important health effects. For example, a high-fat diet can induce liver abnormalities, such as steatosis and inflammation (72), and a recent report suggests that these effects can be eliminated via regulating lipid and glucose metabolism through the consumption of BMOs and *Bifidobacterium longum* subspecies *infantis* in genetically predisposed animal models (55). Considering the interrelated nature of these and other physiological processes, the above studies may represent only a fraction of the metabolic benefits provided by milk OS.

In light of the promising effects of milk OS consumption, industrial interest in marketing these compounds for therapeutic purposes is building. As of January 2019, over 180 US patents have been filed relating to 2′-fucosyllactose alone, and the inclusion of 2′-fucosyllactose as a food ingredient is now commonplace in infant formula (73, 74). The use of 2′-fucosyllactose as an ingredient has prompted a need for large quantities of this OS to be produced synthetically. A multitude of strategies to produce synthetic OS have been designed, using both genetically engineered microorganisms and enzymatic approaches. These strategies, which have been reviewed extensively elsewhere (75), are promising routes by which individual OS could be made available at large scale. These synthetic approaches, in conjunction with the established membrane filtration strategies described above, will likely grow in application to supply the marketplace with OS that can be used as ingredients for therapeutic foods, extending availability of milk OS and their bioactivities to the general public.

CONCLUSION

Growing availability of milk OS for *in vivo* experimentation is uncovering a multitude of unique and previously unknown bioactivities that could be harnessed to alleviate widespread metabolic illnesses. Future studies will likely probe further into the mechanistic details of OS functionalities, as well as evaluate the feasibility of supplementing these molecules into adult diets as therapeutics. The safety and tolerability of isolated milk OS for human consumption were recently evaluated in two independent studies and showed promising results, with even relatively high dosages being well-tolerated (76, 77). These studies could pave the way for the known metabolic impacts of BMOs to be further evaluated in human subjects, including in specialized applications such as infant formula production. Finally, implementing industrial-scale strategies to produce and isolate OS with desired bioactivities will be imperative to the application of OS as therapeutics. Therefore, we should expect continued work to identify factors influencing OS production in dairy cattle, as well as efforts to translate pilot-scale isolation techniques to dairy processing facilities.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

FUNDING

The author acknowledges support from the National Institute of Health award R01AT007079 and from the United States Department of Agriculture CRIS# 8040-51530-056-00D.

ACKNOWLEDGMENTS

The author would like to thank Dr. Daniela Barile for providing feedback on the article content. A portion of the section entitled Analytical methodologies for milk OS is taken from the author's dissertation (75).

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Conflict of Interest Statement: The 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.

frontiers in Nutrition

ORIGINAL RESEARCH
published: 28 March 2019
doi: 10.3389/fnut.2019.00031

Milk Oligosaccharides From Different Cattle Breeds Influence Growth-Related Characteristics of Intestinal Cells

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Oligosaccharides are present in human milk (HMO) in large amounts and in a high variety: Among other functions they are considered to influence the gut microbiota and gut maturation in infants. Due to the large volume of milk available bovine milk oligosaccharides (BMO) may be an alternative source of functional ingredients to potentially mimic HMO functions. Thus, we investigated direct effects of bovine milk oligosaccharides (BMO) from different cattle breeds on proliferation, differentiation and apoptosis in transformed (HT-29 and Caco-2) and non-transformed human intestinal cells (HIE cells). We observed a profound growth-inhibition effect induced by all BMO isolates in HT-29, Caco-2, and HIE cells in a dose-dependent manner. The effects varied not only between cell lines, i.e., HT-29 and Caco-2 cells were more sensitive than HIE cells, but also between the cattle breeds. Regarding the induction of differentiation, BMO induced differentiation only in HIE cells without affecting apoptosis. Cell cycle analysis via flow cytometry showed that growth inhibition was associated with a G2/M arrest in all cell lines. Expression levels detected by quantitative real-time RT-PCR revealed that this G2/M arrest was associated with changes in mRNA expression levels of cyclin A and B. Cyclin-dependent kinase inhibitors $p21^{cip1}$ and $p27^{kip1}$ and the tumor suppressor p53 were only enhanced in HIE cells necessary for arresting cells in the G2/M phase and induction of differentiation. In HT-29 and Caco-2 cells, a loss of p53 expression failed to induce G2/M associated induction of differentiation. The HIE cell specific differentiation induced by BMO was a result of influencing the phosphorylation states of EGFR (epidermal growth factor receptor) and MAP kinase, i.e., ERK1/2 (extracellular signal-regulated kinase 1/2), p38-α, and Akt2 phosphorylation. These results suggest that BMO inhibited intestinal cell proliferation and altered cell cycle dynamics by affecting corresponding regulator genes and mitogen-activated protein kinase signaling. As the development and maturation of digestive and absorptive processes depends on gut differentiation processes, our *in vitro* experiments show that breed-specific BMO are natural substances influencing various parameter which may be important *in vivo* in gastrointestinal development. This, however, needs to be proven in future studies.

Keywords: bovine milk oligosaccharides, BMO, interbreed variation, cell cycle dynamics, differentiation, EGFR-ERK signaling

OPEN ACCESS

Edited by:
F. Javier Moreno, Instituto de Investigación en Ciencias de la Alimentación (CIAL), Spain

Reviewed by:
Sharon Marie Donovan, University of Illinois at Urbana-Champaign, United States
Miryam Amigo-Benavent, University of Limerick, Ireland

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Specialty section:
This article was submitted to Nutrition and Food Science Technology, a section of the journal Frontiers in Nutrition

Received: *24 November 2018*
Accepted: *04 March 2019*
Published: *28 March 2019*

Citation:
Kuntz S, Rudloff S and Kunz C (2019) Milk Oligosaccharides From Different Cattle Breeds Influence Growth-Related Characteristics of Intestinal Cells. Front. Nutr. 6:31. doi: 10.3389/fnut.2019.00031

INTRODUCTION

In the last decade, there has been a tremendous research interest in milk oligosaccharides, driven by the advances in chemical sciences, food technology as well as in chemical-enzymatic synthesis to produce single human milk oligosaccharides (HMO) (1, 2). This interest is primarily based upon the progress in HMO and the increasing number of studies investigating their biological functions (3–7). Currently, two HMO, e.g., 2′FL (Fucosyllactose) alone or in combination with LNnT (Lacto-N-neo-tetraose), which have been industrially produced and which are identical to their human milk counterparts, are already added to infant formula (8, 9). Oligosaccharides in general, whether derived from animals, plants or of synthetic origin, are considered to have an impact on human health, mainly through their prebiotic effects in the gastrointestinal tract. Meanwhile a whole variety of various prebiotics are added to infant formula to imitate their functions (e.g., galacto- and fructooligosaccharides (GOS and FOS), acidic plant derived carbohydrates) (10). It is often concluded that the addition of, for example, GOS and FOS to infant formula would bring them closer to human milk (10, 11). However, these components are not present in human milk (12, 13). There is no structural similarity between such prebiotic GOS/FOS and HMO. So far, no study has been carried out proving that the effects of GOS/FOS on, for example, the immune system are comparable to those of HMO. Due to this discrepancy in structure between both classes of carbohydrates, prebiotic oligosaccharides and HMO, there is currently a great interest in finding alternatives for HMO.

To receive milk oligosaccharides on a large scale which are identical to those in human milk, several strategies are currently applied. Besides chemical-enzymatic synthesis or fermentation strategies, membrane filtration is also used to separate milk oligosaccharide fractions or even individual components to investigate their potential functions in applied science (1, 2, 14, 15). Although the number and the total amount of oligosaccharides in animal milk compared to human milk is rather very low (16) it might be an interesting source to separate a few components due to the huge amount of milk available from cows, goats, or other species. In this context, an interesting aspect to investigate is whether reported differences in milk oligosaccharide compositions between various breeds (17–19) have an influence on functional processes. Hence, various questions, which currently are of great interest from a scientific and a commercial point of view, have to be addressed: Are milk oligosaccharides from various animals promising components to improve the overall health of the recipients? Do single milk oligosaccharides from animals affect the microbial composition and/or activities more efficiently than a mixture of various components and how can health effects be investigated in humans? Which specific oligosaccharides have a direct impact on intestinal or tissue target cells, i.e., on cell maturation, cell surface glycosylation or brain functions?

As we have previously reported on the effects of HMO on proliferation, differentiation and cell signal events (20, 21), the major aim of our current study was to investigate the functional effects of the milk carbohydrate fractions from various cattle breeds using different intestinal cell lines.

Abbreviations: BMO, bovine milk oligosaccharides; HMO, human milk oligosaccharides; 2'FL, 2'Fucosyllactose; GAPDH, Glycerinaldehyd-3-phosphat-Dehydrogenase; GlcNAc, N-acetylglucosamine; LNT, Lacto-N-tetraose; LNnT, Lacto-N-neo-tetraose; GOS, galactooligosaccharides; FOS, fructooligosaccharides; HPAEC-PAD, High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection; NeuAc, N-acetylneuraminic acid; HexNAc, *N*-acetylhexosamine; bHF, German Holstein cattle (Black Pied); rHF, German Holstein cattle (Red Pied); SIM, German Simmental; JER, Jersey; CaCo-2 cells, human colonic carcinoma cells, HIEC, human intestinal epithelial cells; HT-29 cells, human intestinal tumor cells; AP alkaline phosphatase; CDKI, cyclin-dependent kinase inhibitors; EGFR, epidermal growth factor receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; AUC, area under the curve; pMAP, phosphorylated Mitogen Activated Protein Kinases.

RESULTS AND DISCUSSION

Effects of Bovine Oligosaccharides on Proliferation and Differentiation of Intestinal Cells

To address questions related to gut maturation events associated with tissue morphogenetic and cell dynamic changes, we used HT-29 and HIE cells which are intestinal cells with a lower differentiation phenotype, and Caco-2 cells which display characteristics of differentiated epithelial cells (22–25). Regarding proliferation, oligosaccharides from the breeds rHF, bHF, SIM, and JER exerted a pronounced effect in all three cell lines with highly significant interactions ($P < 0.001$) in HT-29, Caco-2, and HIE cells, respectively (**Figure 1**).

The growth inhibition was dose-dependent, albeit with a different magnitude in the three cell lines. Oligosaccharides from JER induced the lowest cell response in all three cell lines which was 17.6 ± 8.14% in HT-29, 16.3 ± 5.78% at the highest concentration (10 mg/mL) in Caco-2 and 17.1 ± 4.77% in HIEC. SIM-derived-oligosaccharides inhibited cell proliferation by 43.2 ± 4.9% (HT-29), 40.9 ± 5.3% (Caco-2), and 25.8 ± 5.6% (HIEC), respectively. Comparing the growth inhibition effect of BMO for the different cell types, HT-29 and Caco-2 cells appeared more sensitive to BMO than HIE cells (**Figure 1**).

Growth inhibition was associated with arresting cells in different cell cycle stages. Flow cytometry analysis showed that, independently of the breed, BMO were able to arrest all intestinal cell lines in the G2/M phase (**Table 1**).

Cell cycle analysis of controls without exposure to BMO revealed that 71.3, 74.9, and 69.3% of HT-29, Caco-2 and HIE cell population was in the G0/G1 phase and 14.5, 12.2, and 20.2% in the G2/M phase. Incubation with BMO led to a reduced cell population in the G0/G1-phase and a higher cell population in G2M-phase compared to controls. However, in all cases the diminished G0/G1- and enhanced G2/M-phase were associated without significant interbreed variations.

As there were no detectable sub-G1 population or caspase-3-activation as markers of apoptosis (data not shown) we investigated the effect of BMO on differentiation (**Figure 2**). We found that treatment with 10 mg/mL BMO derived from rHF, bHF, SIM, and JER enhanced cell differentiation only in HIE cells, but not in HT-29 and Caco-2 cells. Although rHF,

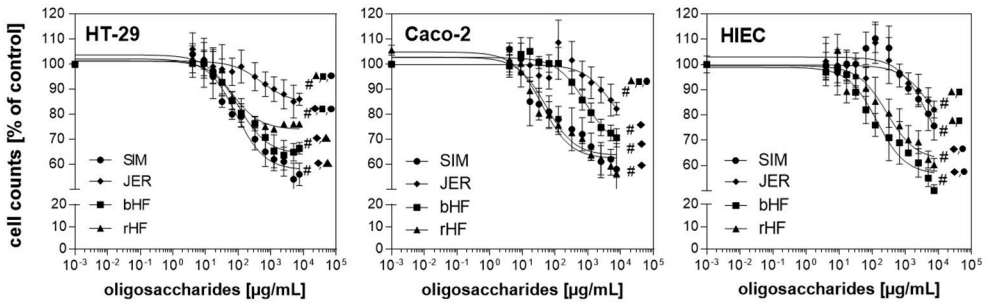

FIGURE 1 | Effect of BMO on the proliferation of intestinal epithelial cells. Dose dependent inhibition effects of BMO from SIM (●), JER (♦), bHF (■), and rHF (▲) on the proliferation of HT-29, Caco-2, and HIE cells. HT-29, Caco-2 (1,500 per well) and HIE (2,500 per well) cells were incubated for 24 h. The cells were then left untreated or treated with BMO at concentrations of 0–10 mg/mL for 72 h. Results were expressed as % of controls (untreated); each value represents the mean with standard deviation ($n = 3$). # indicates significant interbreed variation at 10 mg/mL.

TABLE 1 | Distribution of cell cycle phases after BMO incubation.

		HT-29			
	Control	***rHF***	***bHF***	***SIM***	***JER***
sub G0/G1	0.4%	0.5%	0.3%	0.5%	0.4%
G0/G1	71.3%	49.3%*	53.2%*	56.2%*	48.3%*
S	10.9%	10.3%	10.6%	11.3%	10.4%
G2/M	14.5%	35.5%**	32.4%*	30.3%*	34.3%**
		Caco-2			
sub G0/G1	0.7%	0.5%	0.6%	0.6%	0.4%
G0/G1	74.9%	49.5%*	66.4%*	65.5%*	54.4%*
S	5.4%	9.2%	11.5%	12.2%	10.6%
G2/M	12.2%	39.3%**	34.4%*	31.1%*	37.5%**
		HIEC			
sub G0/G1	1.5%	0.8%	1.6%	1.8%	0.7%
G0/G1	69.3%	48.8%*	50.9%*	52.2%*	47.1%*
S	5.8%	9.8%	6.7%	10.2%	5.4%
G2/M	20.2%	33.8%**	35.4%*	31.1%*	39.3%**

Data are given as % of gated cells in G0/G1-, S-, and G2/M-phases. Cells were left untreated (control) or treated with 10 mg/mL of BMO for 72 h. Growth arrest was determined by flow cytometry with the DNA-staining 7-AAD. Results were expressed as means of % gated cells (n = 3). Significant differences to control cells were accepted at $^{*}P < 0.05$ *and* $^{**}P < 0.01$.

bHF, and SIM-derived BMO were able to increase AP activity to 129.3 ± 6.2, 132.6 ± 8.1, and 173.6 ± 5.9%, respectively, the strongest effect was found using BMO from JER with an induction of differentiation up to 217.9 ± 3.4% compared to control cells. Interestingly, the interbreed variations which were observed at inhibiting HIE cell proliferation was also found at the induction of HIE differentiation for JER- and SIM-derived oligosaccharides.

Taken together, we demonstrated that BMO induced a concentration-dependent growth inhibition in HT-29, Caco-2, and HIE cells by leading to cell arrest in the G2/M phase. However, the effects varied not only between the cell lines but also between oligosaccharides from the four different cattle breeds. HT-29 and Caco-2 cells seemed to be more sensitive to growth inhibition than HIE cells.

Previously, we obtained similar results for growth inhibition and G2/M arrest with HMO as well as with some single oligosaccharides present in both, human and bovine milk (21). Regarding the different effects on the three cell lines, one can speculate that HIE cells are more susceptible to an induction of differentiation than Caco-2 and HT29 cells. In the case of Caco-2 cells, the failure to enhance differentiation can be expected since these cells already represent a more differentiated phenotype reflected by higher basal AP activity (0.609 ± 0.013 ΔE /h/10^6 cell) compared to HT-29 or HIE cells (0.193 ± 0.023 and 0.185 ± 0.005 ΔE /h/10^6 cell, respectively). A phenotype-associated difference in basal AP activity is well-known (26) and supports our hypothesis.

Recently, Holscher et al. (27) confirmed our previous results (20, 22) using slightly different single oligosaccharides at the same concentrations for single HMO (1 mg/mL). Both studies show, for example, that single HMO induce differentiation even in less-differentiated cells. Only in the case of 2′FL there is a difference; here, a reason might be that Holscher et al. investigated the effects of 0.2 and 2 mg/L. In addition, in our studies we used neutral and acidic milk fractions from individual donors whereas Holscher et al. applied pooled human milk obtained from previous studies. Hence, an effect, due to Lewis blood group and secretor specific milk samples on proliferation, differentiation or apoptosis might get lost.

In contrast to our previous results using HMO (20), which induced differentiation in HT-29 and HIE cell, BMO induced differentiation only in HIE cells. The reason for this difference is not yet known, but may be due to the differences in quantity and quality of oligosaccharides present. There is a much higher number of oligosaccharides in human than in bovine milk. HMO contain primarily type 1 components (galactose linked ß1-3 to the subterminal GlcNAc, e.g., in LNT) whereas in BMO primarily type 2 structures (galactose linked ß1-4 to the subterminal GlcNAc, e.g., in LNnT) are present (28). The few oligosaccharides in bovine milk are mostly sialylated (>70%) whereas in human milk acidic components reveal only about 30 % of total oligosaccharides. Another factor responsible for

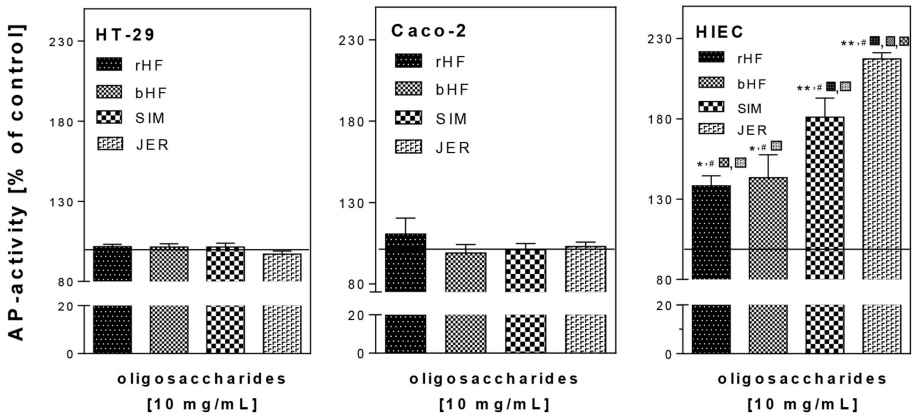

FIGURE 2 | Effect of BMO on the differentiation of intestinal epithelial cells. After incubation with 10 mg/mL BMO, differentiation was determined by measuring alkaline phosphatase (AP) activity after 72 h. Results were expressed as % of control (untreated, solid line); each value represents the mean with their standard error ($n = 3$). Significant differences compared to the untreated control are indicated with $^{*}P < 0.05$ and $^{**}P < 0.01$; # indicates significant interbreed variation at 10 mg/mL.

the different effects of HMO and BMO could be that in human milk only N-acetylneuraminic acid-containing oligosaccharides are present whereas bovine milk contains N-acetylneuraminic acid- as well as N-glycolylneuraminic acid-bearing structures (3, 14, 17, 29).

Effects of Bovine Oligosaccharides on Expression of Cell Cycle Regulator Genes

As shown in **Figure 3** the expression level of cyclin A, a regulator for S/G2-transition, remained unchanged in HT-29 and Caco-2 cells after treatment with BMO, whereas in HIE cells cyclin A expression was stimulated significantly by all BMO. Cyclin B which is responsible for the regulation of the G2/M cell cycle transition, was markedly upregulated in all cell lines after incubation with BMO compared to controls (set to 100%). Cyclin D and E which regulate the entry of cells into and the progression through the G1 phase of the cell cycle remained unchanged in all cell lines after BMO treatment.

In addition, we investigated whether the inhibition of cell cycle progression was accompanied by increased levels of CDKI such as $p21^{cip1}$ and $p27^{kip1}$ and the tumor suppressor gene p53. Both, CDKI and tumor suppressor genes, are able to induce cell cycle arrest in the G1 or G2 phase and/or induce differentiation. Treatment of the undifferentiated cell lines HT-29 and HIE with BMO resulted in an enhanced expression of $p21^{cip1}$ and $p27^{kip1}$ (**Figure 4**). In HT-29 cells, 10 mg/mL of rHF, bHF, SIM and JER-derived oligosaccharides enhanced expression of $p21^{cip1}$ 5.3-, 4.3-, 3.2-, and 5.6-fold, respectively, and that of $p27^{kip1}$ 3.0-, 3.1-, 2.8-, and 4.1-fold, respectively. In HIE cells, rHF-, bHF-, SIM-, and JER-derived oligosaccharides enhanced expression of $p21^{cip1}$ 3.3-, 2.5-, 2.3-, and 4.5-fold, and of $p27^{kip1}$ 2.4-, 2.5-, 2.3-, and 4.0-fold, respectively. In contrast to HT-29 and HIE cells, Caco-2 cells responded only with an increased $p21^{cip1}$ mRNA level but $p27^{kip1}$ levels remained unchanged after BMO exposure.

The observed G2/M arrest in the cells was also based on an increased expression of the CDK inhibitors and of $p21^{Cip1}$ and $p27^{kip1}$ expression. Furthermore, p53, a transcriptional regulator of several cell cycle regulating genes, is able to regulate G1 or G2 transition (30). Interestingly, enhanced p53 mRNA levels were found only in HIE cells after incubation with 10 mg/mL of rHF-, bHF-, SIM-, and JER-derived oligosaccharides.

The effects of BMO observed on proteins responsible for cell cycle progression seemed to be regulated in a similar way as observed with HMO for HT-29 and Caco-2 cells (21). Similar to HMO, BMO from the different cattle breeds induced a p53-independent $p21^{cip1}$ expression with changes of cyclin B. These changes in expression levels were associated with growth inhibition and G2/M arrest. However, our data suggest that BMO-mediated up-regulation of cyclins and CDKIs involves a p53-independent pathway, as HT-29 cells lack functional p53. However, in contrast to our previous observations using HMO, these effects were not associated with an increase in AP activity when HT-29 cells were exposed to BMO (21). Similar to the results obtained by growth-inhibition curves (see above), qualitative and quantitative differences in oligosaccharide pattern between species and even between breeds might be the reason for divergent cell response.

Influence of Signal Transduction Pathways

In order to analyze the effects of BMO on the activation of signal transduction pathways in more detail, the phosphorylation of different growth factor receptors and molecular targets was investigated for HIE cells, representing phenotypical undifferentiated cells, in which BMO were able to induce differentiation, a key event in gut maturation (**Figure 5**). The observed effects on proliferation and differentiation with associated changes in expression levels of cyclins, CDKI and p53 are a consequence of activation or inactivation of different signal cascades. Therefore, we further investigated the influence of the most effective BMO in differentiation (JER-derived oligosaccharides) on the phosphorylation state of several receptors and MAP kinases in HIE cells using protein profiling arrays to detect different phosphorylation events. JER-derived oligosaccharides (10 mg/mL) induced a phosphorylation of the

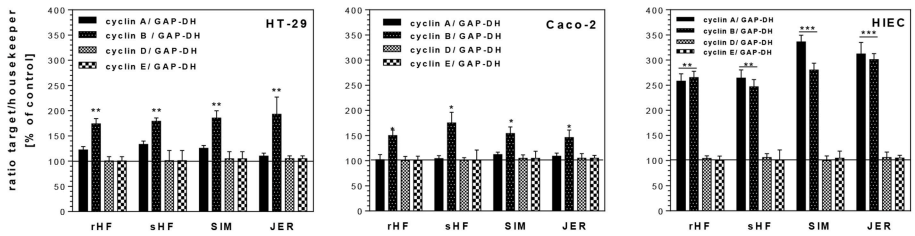

FIGURE 3 | Changes of mRNA expression levels of cell cycle genes (cyclin A, B, D, E) in intestinal HT-29, Caco-2 and HIE cells using GAPDH as housekeeper gene. Cells were treated with 10 mg/mL BMO from rHF, bHF, SIM and JER after reaching a confluency of 30 % over 72 h. Then, mRNA expression levels were determined using the target gene/housekeeping gene ratio by setting the control to 100%. Values are means of the percentage of controls with their standard error ($n = 3$). Mean values were significantly different from those of the control group: $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P<0.001$.

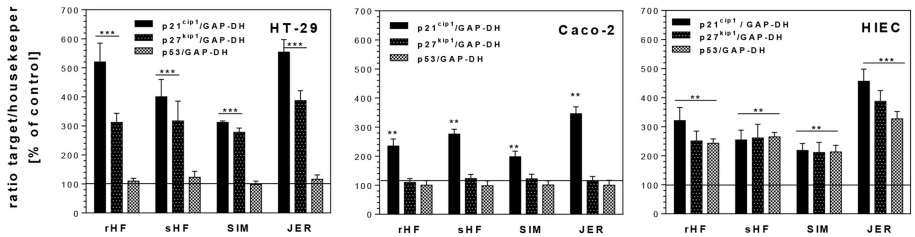

FIGURE 4 | Changes of mRNA expression levels of cell cycle genes $p21^{cip1}$, $p27^{kip1}$, and p53 in intestinal HT-29, Caco-2 and HIE cells using GAPDH as housekeeper gene. Cells were treated with 10 mg/mL BMO from rHF, bHF, SIM, and JER after reaching a confluence of 30% over 72 h. Values are means of the percentage of controls with their standard errors ($n = 3$). Mean values were significantly different from those of the control group: $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$.

ERFR by up to 265 ± 7% compared to untreated control cells (100%) indicating that BMO could interact with the growth factor receptor. This effect was EGFR specific because no other receptor phosphorylation was observed **Figure 5**).

As a consequence of receptor phosphorylation different signal pathways could be induced. Hence, we used a MAPK array to investigate how and to which extent JER-derived oligosaccharides are able to induce downstream events from EGFR signaling. As shown in **Figure 6**, the analysis of these signaling pathways revealed that p38 MAPK, extracellular signal-regulated kinase (ERK) 1 and 2 and protein kinase B (Akt) play a role. Phosphorylation and activation of different p38 MAPK subtypes, especially p38α and p38δ in HIE cells, were induced by JER-derived oligosaccharides (10 mg/mL). In addition, we showed that both PKB and ERK were phosphorylated when cells were treated with oligosaccharides from JER. PKB-β/Akt (Akt2) and Akt pan, two growth factor-regulated protein kinases, and the downstream kinase ERK1 have emerged as critical enzymes in signal transduction pathways involved in cell proliferation and differentiation (31).

Recent studies suggest that the EGFR pathway is not simply a growth promoting signaling pathway, but phosphorylated EGFR (pEGFR) also mediates $p21^{cip1}$ expression and growth arrest or apoptosis (32). From our study two major findings have emerged similar to that found previously with HMO (21): we observed (i) that JER-derived BMO were able to significantly induce EGFR phosphorylation and (ii) we confirmed that the EGFR/Ras/Raf/ERK pathway was involved. Based upon these observations we conclude that BMO-caused differentiation is a consequence of p53-dependent $p21^{cip1}$ expression and stabilization via EGFR and p38 kinase phosphorylation.

In conclusion, we identified that BMO from different cattle breeds were able to induce growth arrest and differentiation of non-transformed HIE cells by modulating EGFR signal pathways, and cell cycle associated gene expression in a similar way as was shown for HMO (20, 21). Whether differences with regard to the magnitude of effects dependent on breed specific BMO (as shown in **Figure 7**) has implications for the intestinal growth regulation in infants is not yet known and requires further investigation. Overall, differences in composition and diversity of milk oligosaccharides will most likely have functional consequences. To proof whether these data translate into the human infant situation, rigorous preclinical and clinical trials would be required to come to a clear conclusion.

MATERIALS AND METHODS

Preparation of Oligosaccharides From Bovine Milk

Milk samples were collected during regular milking from dairy cattle breeds (for milk production) in Germany at the Research Station "Oberer Hardthof" at the Justus-Liebig University Giessen, kindly provided by Prof. G. Erhardt (Institute for Animal Breeding and Genetics). According to the German Animal Welfare Law (released on 05/18/2006) no notification or approval by the Animal Protection Unit of the Regional Council of Gießen (Germany) was necessary for this study. Thus, we

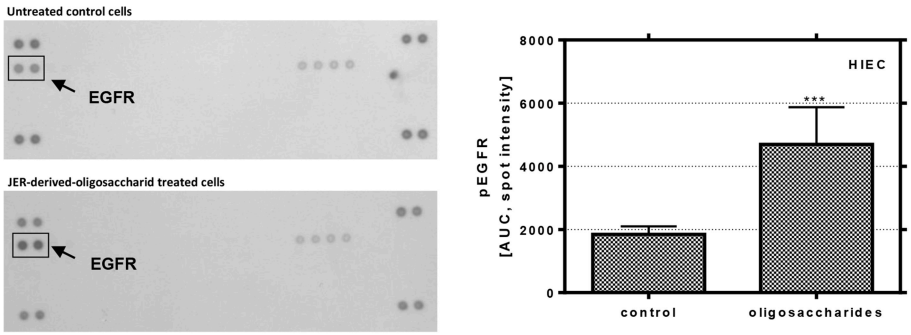

FIGURE 5 | Phosphorylation of EGFR in HIE cells. Cells were treated with 10 mg/mL JER-derived-oligosaccharides for 10 min. Lysate was prepared according to the manufacturer's instructions. Phospho-receptor tyrosine kinase array was used to detect phosphorylation of these receptor tyrosine kinases in HIE cells. The signal was detected by chemiluminescence and the spot intensity is shown. Values are means of the percentage of controls with their standard errors ($n = 2$). Mean values were significantly different from those of the control group (***$P \leq 0.001$) [AUC, area under the curve; EGFR, epidermal growth factor receptor].

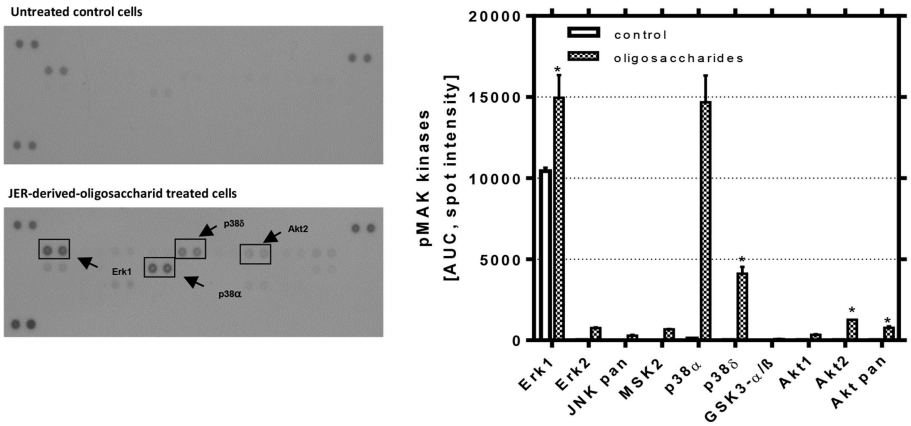

FIGURE 6 | Detection of phosphorylated mitogen-activated protein kinases in HIE cells. HIE cells were treated with JER-derived-oligosaccharides for 30 min. After incubation, 300 μg cell lysate were used for each assay. Array signals from scanned X-ray film images (left: representative blot) were analyzed using image analysis software and expressed as spot pixel density. Values are means with their standard errors ($n = 2$). Mean values were significantly different: *$P \leq 0.05$ and ***$P < 0.001$ [AUC, area under the curve; pMAP, phosphorylated MAPK].

included four breeds, i.e., German Holstein cattle (Black Pied, bHF and Red Pied, rHF), German Simmental (SIM) and Jersey (JER). Cows were milked within the first 48 h of lactation and samples were frozen at −20°C until analysis. BMO were isolated as described previously (20, 21). Briefly, after centrifugation, the lipid layer was removed, and proteins were precipitated from the aqueous phase using ice-cold ethanol. Lactose was removed by gel filtration on Sephadex G-25 (Pharmacia Biotech, Uppsala, Sweden). Pooled oligosaccharide fractions were freeze-dried, their composition was analyzed by high pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using the conditions described previously (33). For cell culture studies bovine oligosaccharides from the different breeds (**Figure 7**) were used at concentrations up to 10 mg/ml in the corresponding culture media with 10% fetal calf serum (FCS), 2 mM glutamine, 100 units/mL penicillin and 100 mg/mL streptomycin (Invitrogen, Karlsruhe, Germany).

Cell Culture

The human colon cancer cell line HT-29 and Caco-2 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The fetal intestinal colon cell line HIEC was generously donated by J.F. Beaulieu (Department of Anatomy and Cell Biology, Faculty of Medicine, Université de Sherbrooke, Sherbrooke, Quebec, Canada). HT-29, Caco-2 and HIE cells were used between passages 45–50, 40–50, and 10–15, respectively. Cells were cultured in 75 cm^2 tissue culture flasks (Renner, Darmstadt, Germany) in RPMI 1640 (HT-29 and HIEC) or DMEM (Caco-2) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine (Invitrogen, Karlsruhe, Germany), 100 units/mL penicillin and 100 mg/mL streptomycin (Invitrogen, Karlsruhe, Germany). The cultures were kept in a humidified atmosphere of 5% CO_2 at 37°C. Cells were passaged at preconfluent densities using 0.05% trypsin and 0.5 mM EDTA (Invitrogen, Karlsruhe, Germany).

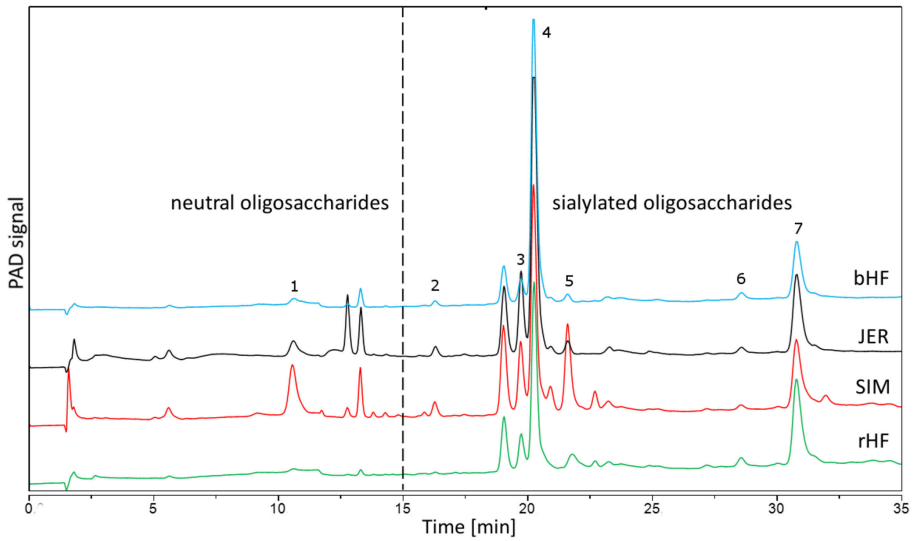

FIGURE 7 | Chromatographic separation using HPAEC-PAD of BMO from four different cattle breeds. BMO from early bovine milk (colostrum) were isolated as described in the Method section. Identified peaks are 1: galactosyl-lactose; 2: *N*-acetylneuraminic acid; 3: 6′-N-acetylneuraminyl-lactose; 4: 3′-*N*-Acetylneuraminyl-lactose; 5: 6′-*N*-acetylneuraminyl-*N*-acetyllactosamin; 6: *N*-glycolyl-neuraminyl-lactose; 7: disialyl-lactose. bHF, German Holstein cattle (Black Pied); rHF, German Holstein cattle (Red Pied); SIM, German Simmental; JER, Jersey.

Measurement of Cell Dynamics

Proliferation of cells was determined after 72 h incubation of adherent cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT)-assay as has been described previously (20). Cell numbers were determined based on a calibration curve using cell counts between 500 and 30,000. Differentiation was determined by alkaline phosphatase activity on 25 cm^2-culture flasks (Renner, Dannstadt, Germany). After having reached 30–40% confluency, cells were incubated for 72 h in the presence or absence (control) of oligosaccharides (pH 7.4) as has been previously described (20). Alkaline phosphatase activity was measured as $\Delta E/h/10^6$ cells and the controls were set to 100%. Cell Cycle Analysis of intestinal cells (HT-29, Caco-2, and HIEC) were measured by flow cytometry. Therefore, cells were seeded at a density of 50,000 onto 6-well culture flasks and allowed to adhere for 24 h. Thereafter, the medium was replaced and incubated for another 24 h in the presence or absence of oligosaccharides. Cell cycle analysis was performed using a FACScan (Becton Dickinson, San Jose, CA, U.S.A.) and the software BD CellQuest™ Pro (Version 1.41.) for data analysis (20, 21).

Measurement of Gene Expression and Signaling Pathways

Total RNA isolation from intestinal cells, cDNA synthesis and real-time PCR were performed as described earlier (21). Messenger RNA expression of cell cycle genes such as cyclins (cyclin A, B, D, and E), CDKI and p53 were determined in relation to the expression of the housekeeping gene GAPDH; results from untreated cells were set at 100% (21). Receptor phosphorylation studies were made with preconfluent (70–80%) HIE cells. Cells were incubated in DMEM with 10% FCS for 24 h. Subsequently, JER-derived oligosaccharides were added in concentrations indicated in the legend of respective figures. The Proteome Profiler™ array—human phospho-RTK array kit to identify the phosphorylation of 42 different RTKs was used according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA) as has been done previously (21). MAP-Kinase phosphorylation studies were made with preconfluent (70–80%) HIE cells. After incubation of intestinal cells with JER-derived oligosaccharides (10 mg/mL), 300 μg of total protein were used for the Human Phospho-MAP array®(R&D Systems; Heidelberg, Germany) according to the manufacturer's protocol (21).

Statistical Analysis

For each variable at least three independent experiments were carried out and the results were expressed as mean values with their standard errors (mean ± SEM) or standard deviation (mean ± SD). Statistical differences were tested by one-way ANOVA with Bonferroni's *post hoc* test and differences were considered significant at $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ to controls or indicate significant interbreed variation at 10 mg/mL with bHF (#a), rHF (#b), SIM (#c), and JER (#d). Two-way ANOVA was used to test significant interactions between concentrations and breeds ($P < 0.001$) in HT-29, Caco-2, and HIE cells, respectively. All analyses were carried out with the GraphPad Software Prism 6.02 (San Diego, CA, USA).

AUTHOR CONTRIBUTIONS

CK, SR, and SK designed the study. SK performed the laboratory work and statistical analysis. SK and SR discussed the interpretation of the data. SK wrote the draft version of the manuscript. SR, CK, and SK had primary responsibility for the final content.

ACKNOWLEDGMENTS

The authors greatly appreciate the excellent technical assistance of Nadine Metz and Cordula Becker. This study was a part of the research focus MEU supported by The Hessen State Ministry for Higher Education, Research and the Arts. Within this research collaboration, Prof. G. Erhardt, Institute of Animal Breeding and Genetics, JLU Giessen, kindly provided us with milk from various cattle breeds.

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