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HIGHLIGHTS IN PEDIATRIC GASTROENTEROLOGY, HEPATOLOGY AND NUTRITION: 2021

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ORIGINAL RESEARCH
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Propofol Sedation by Pediatric Gastroenterologists for Endoscopic Procedures: A Retrospective Analysis

Aya Khalila[1,2], *Itai Shavit*[2,3] *and Ron Shaoul*[1,2]*

[1] Pediatric Gastroenterology and Nutrition Institute, Ruth Children's Hospital, Rambam Health Care Campus, Haifa, Israel, [2] Faculty of Medicine, Technion Israel Institute of Technology, Haifa, Israel, [3] Pediatric Emergency Department, Ruth Children's Hospital, Rambam Health Care Campus, Haifa, Israel

Background: There is a substantial literature on the favorable outcome of propofol administration by non-anesthesiologists for endoscopy in adults; however, very few data are currently available on propofol sedation by pediatric gastroenterologists. Aims: to evaluate the safety of propofol sedation by pediatric gastroenterologists.

Methods: A retrospective chart review of all children who were sedated by pediatric gastroenterologists in three Northern Israeli hospitals over a 4 years period Demographic and medical characteristics and any data regarding the procedure were extracted from patient's records. The main outcome measurements were procedure completion and reported adverse events.

Results: Overall, 1,214 endoscopic procedures for were performed during this period. Complete data was available for 1,190 procedures. All children sedated by pediatric gastroenterologists were classified as ASA I or II. Propofol dosage (in mg/kg) inversely correlated with patient age. The younger the child the higher the dose needed to reach a satisfactory level of sedation ($r = -0.397$, $p < 0.001$). The addition of fentanyl significantly decreased propofol dosage needed to provide optimal sedation, $p < 0.001$. Nine (0.7%) reversible adverse events were reported. All the procedures were successfully completed and all patients were discharged home.

Conclusions: We conclude that our approach is safe in children as it is in adults and can be implemented for children with ASA I, II.

Keywords: sedation, endoscopies, children, safety, non-anesthesiologist administered propofol

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Jorge Amil Dias, Centro Hospitalar de São João, Portugal

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Corentin Babakissa, Université de Sherbrooke, Canada
Javier Martin De Carpi, Hospital Sant Joan de Déu Barcelona, Spain

***Correspondence:**
Ron Shaoul
shaoul_r@012.net.il

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INTRODUCTION

The amount of gastrointestinal endoscopies (GE) performed in childhood has significantly increased over the last two decades, improving both diagnosis, and treatment of pediatric gastrointestinal diseases (1). This has also increased the demand for safe and effective procedural sedation. Pediatric gastrointestinal procedures, such as esophagogastroduodenoscopy (EGD) and colonoscopy require substantial immobilization for successful performance and are uncomfortable and emotionally disturbing for children (2). Some gastroenterologists use light intravenous sedation, typically benzodiazepines, and opioids titrated to levels consistent with conscious

sedation (2–4). Most centers these days regard light sedation as inadequate, and regularly use general anesthesia instead to perform these procedures (2–4). Nevertheless, general anesthesia is only available in a limited number of centers because of shortness of anesthesiologists.

Propofol (2,6-diisopropyl-phenol) is an ultra-short acting sedative agent. It is a phenolic derivative with satisfactory sedative, hypnotic, antiemetic, and amnesic properties. Propofol is highly lipophilic and thus can rapidly cross the blood-brain barrier, resulting in an early onset of action. Regardless of the depth or length of the sedation period, propofol has a short recovery profile (5), there is a conspicuous literature on the favorable outcome of propofol administration by non-anesthesiologists for endoscopy in adults, including a meta-analysis of randomized controlled trials revealing that its use is favored due to rapid onset and offset of action, fast recovery time, and high patient and physician satisfaction (3–17). However, there is no available data on propofol sedation for pediatric gastrointestinal endoscopies by pediatric gastroenterologists.

In the Haifa region of Israel, gastroenterological endoscopies are mainly performed in three medical centers; Rambam Health Care Campus (RHCC), Elisha Medical Center (EMC), and Assuta Medical Center (AMC). In these centers, pediatric gastrointestinal endoscopies are performed by qualified pediatric gastroenterologists, highly skilled and specially trained in pediatric sedation that have been using propofol since 2008 (18). In RHCC, sedations for gastrointestinal endoscopies are performed by a heterogenic group of staff pediatric gastroenterologists and fellows in pediatric gastroenterology. In AMC and EMC sedations are performed by a single qualified pediatric gastroenterologist.

We aimed to retrospectively evaluate the safety and effectiveness of propofol sedation by pediatric gastroenterologists for gastroenterological endoscopies.

METHODS

Study Design

We performed a retrospective chart review of all children who were sedated by pediatric gastroenterologists in RHCC, EMC, or AMC between 1.1.2008 and 31.12.2011.

Demographic and medical characteristics and any data regarding the procedure process were extracted from the electronic records. Data collected included gender, age and weight, type of procedure, medical center (RHCC, AMC, or EMC), sedation medications, and dosages, type of procedure, and any serious adverse events during sedation (SAEDS). Sedation protocol defines SAEDS as: "death, cardiac arrest, endotracheal intubation, hospitalization due to an adverse event, hypoxia (saturation $\leq$ 90%), apnea (discontinuation of breathing), aspiration (coughing or chocking associated with observed gastric contents in the mouth), and laryngospasm (upper airway obstruction with oxygen desaturation caused by closure of the vocal cords), and hypotension (blood pressure below two standard deviations (SDs) of the mean for age and gender) requiring treatment with volume replacement (6)."

Propofol was manually titrated to the desired level of sedation.

The protocol for the research project was approved by a suitably constituted Ethics Committee of RHCC within which the work was undertaken. All human studies have been reviewed by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in an appropriate version of the Declaration of Helsinki (as revised in Brazil 2013), available at http://www.wma.net/en/30publications/10policies/b3/index.html.

Sedation Protocol

Based on Israeli MOH pediatric sedation guidelines, in RHCC, EMC, and AMC all children undergoing gastrointestinal endoscopies are sedated by a pediatric gastroenterologist if the child is older than 24 months, has an American Society of Anesthesiologists (ASA) physical status classification of $\leq$2, and has been fasting for $\geq$6 h. All children under 2 years of age and with ASA score above 2 had been sedated by an anesthetist. Our protocol recommends using propofol with midazolam in separate doses, with midazolam given first to decrease anxiety. All children in the study received 1 mg of midazolam irrespective of weight (all were above 10 kg). The pediatric gastroenterologist and a nurse trained in pediatric sedation were both responsible for patient monitoring. Monitoring includes verifying proper head and neck position and airway patency throughout the procedure, heart rate, and oxygen saturation monitoring. Oxygen supplementation was routinely provided during the procedure. The sedation target was immobilized patient and that the endoscope will be easily inserted. We start with a fixed dose of 1 mg midazolam (serves as anxiolytic drug). It is followed by a slow bolus of fentanyl for colonoscopy in all institutions and for all endoscopic procedures in EMC and AMC). This is followed by 1.0 mg/kg of propofol. We monitor heart rate, oxygen saturation and level of sedation and add boluses of 0.5 mg/kg as needed to achieve and maintain appropriate sedation.

In RHCC, another pediatric gastroenterologist, experienced in sedation is responsible for delivering the sedation. In AMC and EMC the drug is delivered similarly to adult procedures by the endoscopist or by the sedation experienced procedure nurse (third hand).

Patients were discharged from the hospital after at least an hour and after complete recovery, defined by the presence of normal vital signs with the patient being fully awake, without any complains of nausea or vomiting. No known post discharge adverse events were noted.

Written informed consent was signed by the children's caregivers prior to each procedure.

Statistical Analysis

Statistical analysis was performed using SPSS version 21. Quantitative parameters were presented by using means and SDs, and categorical parameters were presented by frequencies and percentage. One way Anova with *post-hoc* tests and *t*-test were used for differences between quantitative parameters in different groups (type of procedure, gender, hospital etc.). Linear correlations between quantitative parameters were used by Pearson correlation. Differences between categorical parameters were used by Pearson chi-square. Linear regression for prediction

TABLE 1 | Patients' characteristics.

	RHCC ($n = 454$)	AMC+EMC ($n = 759$)	*P*-value
DEMOGRAPHIC CHARACTERISTICS			
Age, mean (SD), y	8.7 (5.3)	13.5 (2.6)	0.001
Weight (%), kg	32.25 (19.2)	48.9 (15.2)	0.001
Male (%)	208 (45.7)	365 (48.1)	0.44
ENDOSCOPY			
Upper GI, *n* (%)	353 (77.5)	618 (81.5)	0.1
Lower GI, *n* (%)	43 (9.5)	80 (10.6)	0.55
Upper and lower GI, *n* (%)	60 (7.9)	59 (13)60 (7.9)	0.005
Adverse events, *n* (%)	9 (2)	0 (0)	–

SD, Standard Deviation; GI, Gastrointestinal; RHCC, Rambam Health Care Campus; AMC, Assuta Medical Center, EMC, Elisha Medical Center.

propofol per weight was used with three independent parameters (type of procedure, gender, type of hospital) $P < 0.05$ was consider as significant.

RESULTS

Overall, 1,214 diagnostic endoscopic procedures have been performed during this period. Four hundred and fifty-five (37.5%), 365 (30%), and 394 (32.5%) endoscopies were performed in RHHC, AMC and EMC, respectively. Complete data was available for 1,190 procedures. Nine hundred and seventy one (80%) were upper endoscopies, 123 (10.1%) were colonoscopies and 119 (9.9%) were combined upper-and-lower endoscopies. Seventy one sedations have been carried out by an anesthetist. Demographic and medical characteristics are shown in **Table 1**.

Propofol Dosage

Mean propofol dosage is reported in **Table 2**. Propofol dosage was significantly higher in RHCC compare to AMC and EMC (4.06 vs. 2.28 mg/kg, $p < 0.001$).

Propofol dosage (in mg/kg) correlated with patient age. The younger the child the higher the dose needed to reach a satisfactory level of sedation ($r = -0.397$, $p < 0.001$, **Table 2**; **Figure 1**). We did further analysis according to the procedure type and the findings remained the same. $r = -0.588$, $p < 0.0001$ for colonoscopy, $r = -0.487$, $p < 0.0001$ for upper endoscopy and $r = -0.634$, $p < 0.0001$ for combined procedures. Interestingly, females required a less dosage of propofol than boys in order to reach an appropriate level of sedation, $p = 0.006$.

Propofol dosage was significantly higher in combined upper-and-lower endoscopy compare to upper endoscopy and lower endoscopy ($p < 0.001$, for all comparisons, **Table 2**).

Propofol dose/kg was significantly higher during anesthetist presence compared to pediatric gastroenterologist, 5.69 ± 2.78 kg vs. 2.77 ± 1.39 mg/kg, $p < 0.001$, respectively. This trend can be seen in **Figure 2**. Propofol dose/kg was also significantly higher in RHCC hospital compared to the other hospitals (**Figure 3**).

TABLE 2 | Propofol dosage (mg/kg) in the different settings.

	Propofol dosage (mg)/weight (kg)		
	N	Mean ± SD	*P*-value
GENDER			
Male	566	3.082 ± 1.67	$P = 0.006$
Female	624	2.818 ± 1.618	
Total	1,190	2.944 ± 1.65	
HOSPITAL			
RHCC	444	4.056 ± 1.94	$P < 0.001$
AMC + EMC	746	2.282 ± 0.96	
Total	1,190	2.944 ± 1.65	
ENDOSCOPY TYPE			
Upper	951	2.683 ± 1.39	[a]$p < 0.001$
Lower	120	3.595 ± 2.11	[b]$p < 0.001$
Upper/lower	118	4.398 ± 2.08	[c]$p < 0.001$
Total	1,189	2.944 ± 1.65	
Anesthesiologist	71	5.69 ± 2.78	$p < 0.001$
Gastroenterologist	1,119	2.77 ± 1.39	
COMPLICATIONS			
No	1,181	2.928 ± 1.64	$p < 0.001$
Yes	9	4.986 ± 1.68	

[a]*Significant differences between EMC hospital vs. RHCC, hospital.*
[b]*Significant differences between EMC hospital vs. AMC, hospital.*
[c]*Significant differences between RHCC hospital vs. AMC, hospital.*

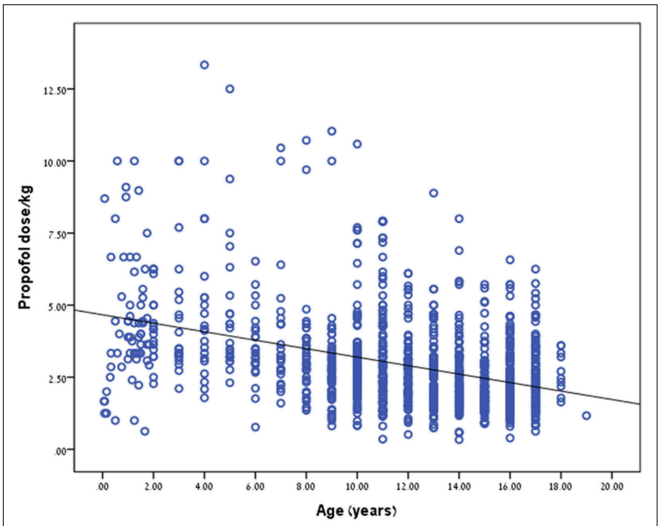

FIGURE 1 | Relation between patient age and propofol dose (mg/kg).

The mean midazolam dose was 0.0385 ± 0.03220 and the mean fentanyl dose (864 patients) was 0.001434 ± 0.0006189 mg/kg.

SAEDS

Nine (0.7%) SAEDS were reported, all in RHCC. No deaths were reported, no patient was reported to be admitted due to an adverse reaction, and no patient required placement of endotracheal tube. All the procedures were successfully

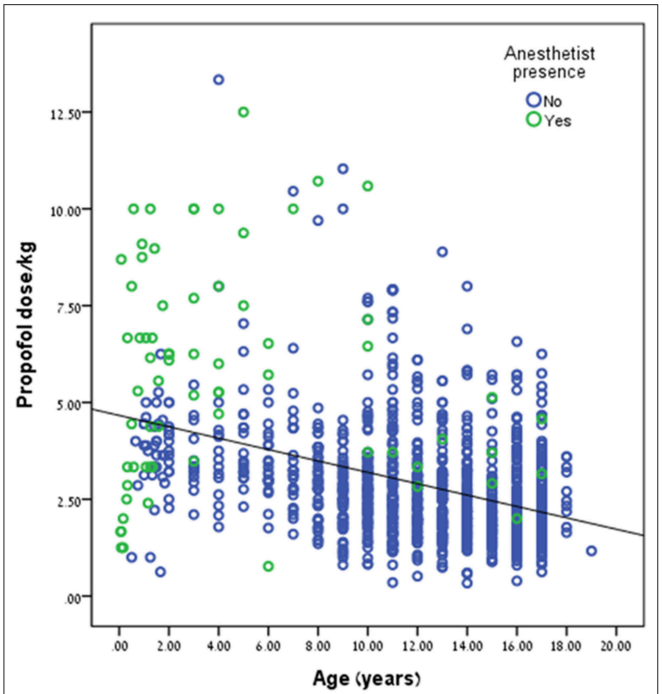

FIGURE 2 | Relation between presence of anesthetist and propofol dose.

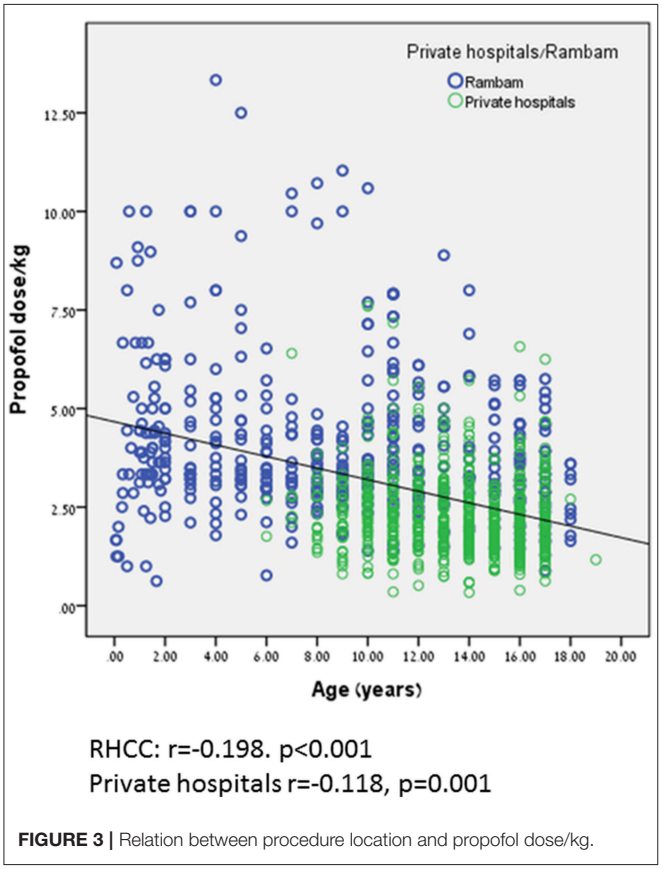

FIGURE 3 | Relation between procedure location and propofol dose/kg.

completed and all patients were discharged home. No significant hypotension, or cardiac arrhythmias were reported. In one case, an 8 years old developed laryngospasm with oxygen desaturation during an upper endoscopy. The sedation for this procedure was given by an anesthetist. He was successfully treated with bag-mask ventilation and dexacort and was discharged asymptomatic after few hours of observation. Eight children (7 upper endoscopies and 1 lower endoscopy) experienced short episodes of oxygen desaturation that resolved with repositioning of the airway. Children who experienced SAEDS received higher doses of propofol 4.9 ± 1.68 vs. 2.92 ± 1.64 mg/kg, $p < 0.001$.

The Effect of Fentanyl on Propofol Dose

In order to examine the impact of combined sedation with fentanyl, propofol, and midazolam on propofol dosage, we compared between all children who received combinative sedation of the three sedative agents and children who did not received fentanyl. Overall, 864 (852 for whom complete data was available) children received combinative sedation of propofol and fentanyl. The addition of fentanyl significantly decreased propofol dosage needed to provide optimal sedation, $p < 0.001$ (**Table 2**).

DISCUSSION

The primary goals of most sedation regimens for pediatric endoscopic procedures areto ensure relaxed and safe atmosphere for the patient throughout the procedure. Secondary and often desirable goals of sedation are to effect peri-procedural amnesia, maximize procedural efficiency, minimize recovery times, and maintain cost-effectiveness (4). Although general anesthesia (GA) is considered safe and effective in providing comfort and amnesia, GA requires expertise and has been viewed as not being cost effective for pediatric endoscopies (7). This together with lack of trained anesthesiologists pushes toward the practice of sedation by non-anesthesiologist providers during endoscopies. The use of IV sedation by pediatric endoscopists before the introduction of propofol was associated with a high risk for agitation, which adversely affect the quality of procedures for both patients and clinical staff (4). One way of lowering the incidence of patient agitation during pediatric endoscopies is to use propofol sedation, either as an isolated IV administration or in combination with other sedative drugs. Propofol has been shown in multiple studies to be highly effective at inducing sedation in children who are undergoing both upper and lower endoscopy, and provides excellent amnesia for the procedure (8–10). The introduction of propofol into adult practice and the data gained on its safety in pediatric procedures and pediatric emergency departments (11) has pushed toward its use in pediatric gastroenterology suites. This is the largest series of propofol administered by pediatric gastroenterologists ever reported to our knowledge.

Heuss et al. (12) reported that 43% of 180 Swiss endoscopists who replied to the survey use propofol without the assistance of

an anesthesiologist regularly, mainly in a hospital setting. They had performed a total of 82,620 procedures. The morbidity in this group of patients was 0.19%, with no cases of mortality.

In a meta-analysis of 12 original studies including 1,161 adult patients of whom 634 received propofol, and 527 received midazolam, meperidine, and/or fentanyl. The pooled odds ratio with the use of propofol for developing hypoxia or hypotension for all the procedures combined was 0.74 (95% confidence interval [CI], 0.44–1.24); for EGD, 0.85 (95% CI, 0.33–2.17) and for colonoscopy, 0.4 (95% CI, 0.2–0.79) (13). A more recent meta-analysis (14) on 1,798 adult patients, of whom 912 received propofol only and 886 received traditional sedative agents supported these findings and concluded that propofol is safe and effective for gastrointestinal endoscopy procedures and is associated with shorter recovery and discharge periods, higher post-anesthesia recovery scores, better sedation, and greater patient cooperation than traditional sedation, without an increase in cardiopulmonary complications.

Based on that several position statements (15, 16) the safety profile of non-anesthesiologist administered propofol (NAAP) is equivalent to that of standard sedation with respect to the risks of hypoxemia, hypotension, and bradycardia for both upper endoscopy and colonoscopy. For EGD, colonoscopy, ERCP, and EUS, the time for sedation induction is shorter with NAAP than with standard sedation, recovery time for these procedures when using NAAP is shorter than for standard sedation with a narcotic and a benzodiazepine. Patient satisfaction with NAAP is equivalent or slightly superior to that with standard sedation.

Larsen et al. (17) reported the safety of propofol sedation by a pediatric intensivist for 4,716 pediatric outpatient procedures of which 2,332 (49%) were gastrointestinal. 56% were <10 years. For this group they had 355 minor complications (15.2%) (transient requirement of oxygen by nasal cannula or positive pressure ventilation by mask, airway repositioning by jaw thrust, or oropharyngeal suctioning to improve oxygen saturation) and one major complication (0.04%) at the 1–10 years age group in a child with glycogen storage disease and adenoid hypertrophy. They concluded that propofol sedation by a pediatric intensivist is a safe sedation technique in the pediatric outpatient setting. Barbi et al. (18) assessed the safety and efficacy of procedural sedation with propofol by non-anesthesiologist pediatric sedation unit using intravenous propofol. Transient desaturation resolving spontaneously occurred in 134 (12.7%) of 1,059 patients. Major desaturation requiring a short course of ventilation occurred in 4 (0.8%) of 483 patients undergoing upper endoscopies. The same group prospectively reported 3 years later (19) the use of propofol for 811 upper gastrointestinal endoscopy in children (ASA grades I–II), administered by specially trained pediatricians. None of the patients required intubation. Stridor with signs of upper airway obstruction occurred in 14 of the 811 procedures (1.7%). Major desaturation requiring a short course of ventilation occurred in six procedures (0.7%), and transient desaturation that resolved spontaneously occurred in 97 of the procedures (12%).

Van Beek et al. (20) recently reviewed 6 RCTs ($N = 561$ procedures) and 4 non-RCTs ($N = 3,322$ procedures) examining the safety and/or effectiveness of propofol based pediatric sedation. The majority of published propofol sedations (3,420/3,883; 88.1%) were performed by non-anesthesiologists [pediatric intensivists (8, 17) or specifically trained pediatricians (18, 19)]. They concluded that Propofol-based procedural sedation is safe. On a total of 3,883 reported propofol-based sedations, major respiratory complications like total airway obstruction, deep hypoxia, or apnea occurred 11 times (0.3%). They emphasize that mild respiratory events occur frequently and major complications may happen rarely, but adverse events do not occur more frequently compared with other sedation regimens. No cases of intubation, resuscitation, permanent sequelae, or death were reported. This is consistent with our findings.

Milius et al. (21) found that the mean dose of propofol required for female patients was 3.7 vs. 3.4 mg/kg for males ($p = 0.3$). In our study we observed the opposite trend (girls needed significantly less propofol dose/kg to achieve same sedation level). Another finding in their study was that the mean doses of propofol for patients ≤9 years, 10–12 years, and >12 years were 3.2, 3.9, and 3.9 mg/kg, respectively ($p = 0.25$). We noted an opposite trend in our study (higher doses/kg for younger patients). These opposite trends may be attributed to a much larger population in our study. The higher propofol dose/kg in procedures performed by anesthetist may be attributed to less experience in gastrointestinal sedation and dealing with younger and more complicated patients. The differences noted in propofol dose/kg between RHCC and the other hospitals are probably related to older age in AMC and EMC hospitals (although difference is still noted after correction for age), a single highly experienced endoscopist compared to mixed population of residents and young seniors performing the procedures in RHCC.

The combination of propofol with midazolam and fentanyl has been shown to decrease the amount of propofol and/or decrease recovery time in adults (22, 23). It has been shown to improve sedation quality in children. (10) We routinely add midazolam to our sedation and the addition of fentanyl has been shown to significantly reduce propofol dose in our study as well.

Since propofol has a narrow therapeutic range and there is no specific antagonist available, the administration of propofol had been restricted primarily to anesthesiologists and trained nurse anesthetists in order to manage airway in emergency (24). However, propofol has been noticed on account of the rapid time to onset and recovery time, in addition to the better or similar patient satisfaction (25, 26). Propofol has also been proven to reduce post-procedural hypoxemic events (27). Some recent studies suggest that propofol can be safely administered to children by non-anesthesiologists who specifically trained to follow established safety guideline (17–20, 28, 29).

The main limitations of the study are its retrospective nature. Nevertheless, all adverse events (minor and major) are well documented in patient's chart.

In their recently published review on sedation for gastrointestinal endoscopy in children by non-anesthesiologists, Orel et al. (30) denote that in many countries, including a majority of European countries and in parts of the United States, the limited availability of anesthesiology teams and limited

organizational considerations represents a medical dilemma and an alternative should be sought. They provide evidence for sedation schemes, including propofol, which could be safely and efficiently performed by non-anesthesiologists as long as they are adapted to international, national and local legislation and institutional practice.

We practice as suggested by Bartkowska-Sniatkowska et al. that children with congenital defects and serious coexisting diseases (ASA $\geq$ III) must be managed by pediatric anesthesiologists (31). We conclude that our approach is safe in children as it is in adults and should be implemented for children with ASA I, II in countries suffering from anesthetists shortage.

AUTHOR CONTRIBUTIONS

AK contributed to this submitted work by reviewing patient charts and collecting data, and analyzing and interpreting the results. She gave final approval of the version to be published. IS contributed to this submitted work by assisting with sedation protocols, helped with writing of the manuscript. He gave final approval of the version to be published. RS contributed to this submitted work by designing the study and the variables to be investigated and by guiding the process. He wrote the manuscript.

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Psychometrics of the Functional Oral Intake Scale for Infants

You Gyoung Yi[1,2] *and Hyung-Ik Shin*[2]*

[1] Department of Rehabilitation Medicine, Veterans Medical Research Institute, Veterans Health Service Medical Center, Seoul, South Korea, [2] Department of Rehabilitation Medicine, Seoul National University Hospital, Seoul National University College of Medicine, Seoul, South Korea

This study aimed to investigate the reliability and validity of the Functional Oral Intake Scale (FOIS) for infants. Infants (age, <1 year) who underwent a videofluoroscopic swallowing study (VFSS) were included in this retrospective study. Their nutrition records at the time of the VFSS were separately evaluated by two raters using the five-point FOIS for infants. Categorical swallowing and aspiration impairment scale data were also obtained from the VFSS. The inter-rater reliability of the FOIS for infants was high (95.5% absolute agreement) among the 201 evaluated infants, and this scale was significantly correlated with aspiration severity in the VFSS. We also investigated whether infants with partial oral feeding (POF) at the FOIS evaluation had achieved full oral feeding within 1 year of the evaluation and used this information to estimate whether the caloric contribution, as well as consistency of oral feeding, affected the feeding outcomes. This analysis included 33 infants who were receiving both oral and tube feeding (i.e., POF). Among them, 26 infants achieved full oral feeding (FOF) without tube feeding after 1 year. Their initial contribution from oral feeding was higher than that in infants who still maintained POF after 1 year (28.46 ± 22.79 vs. 6.00 ± 5.45%, $p < 0.001$). The five-point FOIS for infants, which reflected the expansion of their oral diet with growth, had adequate reliability and validity. The caloric contribution as well as consistency of oral feeding could be used to distinguish FOIS levels 2 and 3, which correspond to the POF status in infants.

Keywords: eating abilities, infant, functional oral intake scale, videofluoroscopic swallowing study, oral feeding, nutrition

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Université de Sherbrooke, Canada

***Correspondence:**
Hyung-Ik Shin
hyungik1@snu.ac.kr

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INTRODUCTION

Interventions for infants with dysphagia, such as environmental modifications (1), oral-motor stimulation (2), altered feeding routines (3), and neuromuscular electrical stimulation (4), have attracted increased attention both in clinical and research perspectives. Although such infants often require tube feeding to achieve a satisfactory caloric intake, this practice may lead to later feeding difficulties (5). Especially, in the case of starting tube feeding during the first year of life, it has been reported that the feeding outcome was poor although the pharyngeal phase of swallowing function is well preserved. Therefore, oral feeding in this era of tube feeding is recommended and encouraged (6). These earlier findings underscore the current paucity of and need for validated tools to measure the effects of these interventions and describe the swallowing status of infants.

The Functional Oral Intake Scale (FOIS, **Table 1**) was initially developed for the clinical documentation of changes in the functional oral intakes of liquids and foods by stroke patients (7).

TABLE 1 | The functional oral intake scale according to Crary et al. (7).

Level 1	Nothing by mouth
Level 2	Tube-dependent with minimal attempts of food or liquids
Level 3	Tube-dependent with consistent oral intake of food or liquids
Level 4	Total oral diet of a single consistency
Level 5	Total oral diet with multiple consistencies but requiring special preparations or compensations
Level 6	Total oral diet with multiple consistencies without special preparation but with specific food limitations
Level 7	Total oral diet with no restrictions

TABLE 2 | The modified functional oral intake scale for infants according Coppens et al. (15).

	Intake
Level 1	Nothing by mouth
Level 2	Tube dependent with minimal attempts of food or liquids
Level 3	Tube dependent with consistent oral intake of food or liquids
Levels 4–6	Expansion of oral diet not reached[a]
Level 7	Expansion of oral diet reached[a]

[a]*Normal expansion of oral diet was considered reached when introduction of solid foods in pureed form started before 9 months of age and the introduction of mashed foods and soft lumps started before 12 months of age.*

This seven-point observer rating scale is considered a reliable and valid tool that can be applied without placing an additional burden on the patient. In adults with dysphagia, the FOIS has been reported to correlate significantly with the Food Intake Level scale (8), swallowing item of the Functional Assessment Measure (9), Mann Assessment of Swallowing Ability, modified Barthel Index, modified Rankin scale, and dysphagia and aspiration during a videofluoroscopic swallowing study (VFSS) (7). Despite the wide use of the FOIS to evaluate dysphagia and assess oral intake recovery in adults (10), it has not been validated for use in infants.

The direct application of the FOIS to infants is challenging, as they are developing rapidly and will experience an expansion of the oral diet with age (11–13). Additionally, it can be difficult to distinguish FOIS level 2 (tube feeding with minimal attempts of oral feeding) from FOIS level 3 (tube feeding with consistent oral feeding) because consistent but very small amounts of oral feeding are possible during the period of tube feeding (14). Accordingly, Coppens et al. modified the FOIS (**Table 2**) for the evaluation of infants subjected to esophageal atresia repair by reducing the FOIS levels from seven to five stages to reflect the food expansion status (15). However, the authors did not report the validity or reliability of this modified scale. Therefore, the present study aimed to investigate the reliability and validity of this modified FOIS for infants. Additionally, we evaluated whether the oral feeding amount and frequency could be used to distinguish FOIS levels 2 and 3.

MATERIALS AND METHODS

All study-related procedures were performed in accordance with the ethical standards of the institutional and/or national research committee and the 1964 Declaration of Helsinki. Ethical approval for the study was obtained from the Seoul National University Hospital Institutional Review Board (IRB) (No. 1807-189-963), which waived the requirement for informed consent due to the retrospective nature of the study. The following inclusion criteria were applied to potential subjects: (1) participation in the VFSS to evaluate a swallowing disorder at $\leq$1 year of age between 2011 and 2017 and (2) recording of the dietary status at the time of the VFSS by a nutritionist.

FOIS for Infants

A seven-point ordinal FOIS has been validated in adults (**Table 1**) (7). As noted in the Introduction, a modified five-point version of the FOIS was developed to account for normal infant development (**Table 2**) (16). At FOIS levels 1, 2, and 3, the same criteria as those for adults were used in this study. However, we divided full oral feeding (FOF) into two categories: (1) achievement of oral diet expansion, the initiation of pureed foods before 9 months, and the initiation of mashed foods and those with soft lumps before 12 months as normal developmental stages; and (2) no achievement of this oral diet expansion.

Inter-rater Reliability

The infants' caregivers were interviewed by a nutritionist, who recorded the type, amount, and consistency of food and liquid intakes, tube dependency, and total nutrient intake. Two occupational therapists with >2 years of experience in swallowing therapy retrospectively reviewed the nutritionist's medical records and assigned FOIS levels.

Validity

Cross-validity was determined by comparing the infantile FOIS scores with the categorical ratings of swallowing impairment/aspiration severity and on the basis of the presence of swallowing impairment/aspiration determined by the VFSS (16). These tools were also used to validate the original FOIS (7). The swallowing impairment scale score was rated as 5 (normal) in the absence of a swallowing abnormality and as 1 (complete) if there was no response to a food stimulus. The aspiration impairment scale score was rated as 5 (normal) if the contrast material did not enter the true vocal cord and as 1 (complete) if the infant showed frank aspiration without reflex coughing (**Table 3**).

Nutritional Contribution of Oral Feeding in Infants With Partial Oral Feeding

For infants with partial oral feeding (POF), the calorie contribution of oral feeding to the total caloric intake was estimated based on the same records used for the FOIS evaluation. Calories were calculated using the web version of CAN-Pro 5.0 software (http://www.kns.or.kr/English/index.asp, The Korean Science and Technology Center, Gangnam-gu, Seoul, Korea), which was developed by the Korean Nutrition Society for the nutritional evaluation of individuals or groups. If any food was not registered in the program, calories were calculated from the information printed on the product container. We also

TABLE 3 | Videofluoroscopic diagnostic criteria for dysphagia and aspiration, adopted from Mann et al. (16).

SWALLOWING IMPAIRMENT (DYSPHAGIA)	
Normal	No swallowing abnormality detected
Mild	Slight delay in bolus control, initiation of swallow, or transport, resulting in some stasis of material without laryngeal penetration
Moderate	Moderate delay in bolus control, initiation of swallow, or transport, resulting in coating or stasis of materials within the oral cavity and/or pharynx, slight laryngeal penetration, or trace aspiration of thin liquid only
Severe	Substantial delay in bolus control, initiation of swallow, and transport; significant (>10% of bolus) penetration and/or aspiration of one or all consistencies
Complete	No response to food stimulus; initiation of the swallow sequence is not obtained over several trials
ASPIRATION	
Normal	No entry of contrast material through the true vocal cords
Mild	Trace entry of contrast materials through the vocal cords
Moderate	Entry of <10% of the bolus through the true vocal cords
Severe	Entry of >10% of the bolus through the true vocal cords
Complete	Frank aspiration of materials through the vocal cords without an observable reaction by the patient

investigated whether infants with POF at the FOIS evaluation had achieved FOF within 1 year of the evaluation and used this information to estimate whether the caloric contribution, as well as consistency of oral feeding, affected the feeding outcomes.

Statistics

For the five-point FOIS for infants, Cohen's κ and Cronbach's α coefficient were calculated as measures of the inter-rater reliability between the two evaluators. To assess cross-validity, Spearman's ρ-test was used to assess correlations between the FOIS for infants with swallowing impairments and aspiration severity ratings. We used Cramer's V (dichotomized data) to determine the association between the FOIS for infants and the presence or absence of swallowing impairment and aspiration.

Infants with POF were stratified according to whether they achieved FOF or not at 1 year after the evaluation, as determined by the caloric contribution of the oral intake at the time of the initial FOIS evaluation. Differences between these two groups were analyzed using an independent t-test. $P < 0.05$ was considered statistically significant. Analyses were performed using SPSS ver. 23.0 (IBM Corporation, Armonk, NY, USA).

RESULTS

Subjects

Data were obtained from 201 infants (mean age: 199 days, range: 22–364 days) who underwent a VFSS between 2011 and 2017. The baseline characteristics and main diagnoses of the subjects are presented in **Table 4**. Brain lesions were found in 63 infants, which included hypoxic ischemic encephalopathy, encephalitis, intracerebral hemorrhage, brain tumor, corpus callosal dysgenesis, and hydrocephalus. Myopathy/motor neuron disease including spinal muscular atrophy, mitochondrial myopathy, myotubular myopathy, Fukuyama congenital muscular dystrophy, and congenital muscular dystrophy was found in 21 infants. The non-oral feeding, POF, and FOF groups did not differ significantly in age at the time of FOIS evaluation. VFSS findings with aspiration of liquid were found in 61 infants among 201 infants. The swallowing impairment scale (**Table 3**) scores ranged from 1 to 5 with a median of 4 (interquartile range: 3–4) with 38 infants having a score of 5, 78 with a score of 4, 53 with a score of 3, 31 with a score of 2, and 1 with a score of 1.

Inter-rater Reliability of the FOIS for Infants

The two occupational therapists achieved a high level of absolute agreement (95.5%) when applying the FOIS for infants (**Table 2**), as shown in **Table 5** ($\kappa = 0.935$; intraclass correlation coefficient [ICC] = 0.996; 95% confidence interval [CI]: 0.995–0.997). The main disagreements were observed between FOIS levels 2 and 3 ($n = 3$) and levels 4 and 5 ($n = 5$).

Validity

This study identified significant associations between the subject's level on the FOIS for infants and the presence ($p = 0.014$, $V = 0.249$) and severity ($p = 0.001$, $r = 0.229$) of aspiration during the VFSS. The infantile FOIS ratings correlated significantly with the severity ($p = 0.040$, $r = 0.145$), but not the presence of dysphagia ($p = 0.188$, $V = 0.175$).

Nutritional Contribution of Oral Feeding in Infants With POF

This analysis included 33 infants who were receiving POF at the time of the VFSS and for whom nutritional records at 1 year after the VFSS were available (**Figure 1**). Among them, 26 infants achieved FOF after 1 year, and their mean nutritional contribution from oral feeding at the time of VFSS was 28.46 ± 22.79%, which was higher than 6.00 ± 5.45% in the seven infants who maintained a POF status ($p < 0.001$, **Figure 2**).

DISCUSSION

There is a need to aid clinicians in describing the feeding status and measuring outcomes for the management of infants with dysphagia. For example, therapeutic interventions such as oral sensorimotor stimulation for infants with oral hypersensitivity (17, 18) and fluid thickening to minimize aspiration symptoms (19, 20) necessitate describing the feeding outcome of the infants.

Accordingly, studies have validated the Neuromuscular Disease Swallowing Status Scale for children and adults (21, 22) and the Eating and Drinking Ability Classification System for children with cerebral palsy over a 3-year period (23, 24). However, in patient groups other than those described above, the adult version of the FOIS has been modified for the pediatric population.

Dodrill et al. (25) modified the FOIS to describe the swallowing function of infants/toddlers by replacing the levels

TABLE 4 | Characteristics of subjects at the time of the videofluoroscopic swallowing study.

Characteristics	Eating abilities in infants		
	Non-oral feeding (*n* = 80)	Partial oral feeding (*n* = 44)	Full oral feeding (*n* = 77)
Female sex (%)	38 (47.5)	21 (47.7)	36 (46.8)
Age (range), days	171 (22–364)	233 (65–349)	210 (53–364)
Main diagnosis, *n* (%)			
Brain lesion	25 (31.3)	8 (18.2)	30 (39.0)
Myopathy/motor neuron disease	10 (12.5)	5 (11.4)	6 (7.8)
Gastrointestinal	6 (7.5)	6 (13.6)	7 (9.1)
Cardiac	4 (5.0)	3 (6.8)	5 (6.5)
Otolaryngology	7 (8.8)	4 (9.1)	10 (13.0)
Metabolic	6 (7.5)	0 (0)	1 (1.3)
Pulmonary	4 (5.0)	3 (6.8)	4 (5.2)
Immunologic	0 (0)	0 (0)	2 (2.6)
Unknown	1 (1.3)	0 (0)	1 (1.3)
Syndrome	17 (21.3)	15 (34.1)	11 (14.3)
Pierre Robin Syndrome	1	2	2
Kabuki syndrome	1	1	
Zellweger syndrome	1		1
Beckwith-Weidemann syndrome			1
Schinzel-Giedion syndrome			1
VACTERL syndrome	1		
Cornelia de lange syndrome		2	
Patau syndrome	1		
Sotos syndrome			3
Noonan syndrome	1		
Down syndrome		1	1
Miller-Dieker syndrome	1		1
Mobius syndrome		1	
CHARGE syndrome	2	2	
Treacher Collins syndrome	1	1	
Russel Silver syndrome	1		
Prader Willi syndrome	2		
Goldenhar syndrome	1	2	
CATCH 22 syndrome	3	1	
Smith-limli-opitz syndrome		1	
Wolf Hirschhorn syndrome			1
Mosaic 22q13 deletion syndrome		1	

indicating single/multiple consistency (level 4 and 5, respectively, in the adult version of the FOIS) with levels indicating requirement of modified liquids/solids (level 4 and 4.5, respectively, in the modified FOIS for infants). Strychowsky et al. utilized this version of the FOIS to describe the swallowing dysfunction among toddlers with laryngeal cleft (26). Christiaans et al. (4) also modified the original version of the FOIS by removing the level 4: total oral diet of a single consistency, in their report regarding the effectiveness of neuromuscular electrical stimulation in children with dysphagia. Later on, Baxter et al. applied the scale in children with esophageal atresia and tracheoesophageal fistulas (27).

However, these modified FOIS versions for the pediatric population have never been validated and tested for reliability. Additionally, these scales were proposed and used for young children as well as infants. Since oral diet expansions occur during the infantile period, the functional oral intake of infants should be assessed separately from that of young children. Therefore, we selected the modified FOIS that is specific to infants, proposed by Coppens et al. (15), and verified the validity and reliability of the scale. To our knowledge, this is the first study to validate the scale that was modified to measure food or liquid consumption by infants.

Inter-rater Reliability and Validity

In this study, we observed a high level of inter-rater reliability for the FOIS for infants, which was similar to that in other studies.

TABLE 5 | Inter-rater reliability of the FOIS for infants.

	Rater 2					
Rater 1	**1**	**2**	**3**	**4**	**5**	**Total**
1	79	1[a]	0	0	0	80
2	0	2	1	0	0	3
3	0	2	39	0	0	41
4	0	0	0	7	3	10
5	0	0	0	2	65	67
Total	79	5	40	9	68	201

Shaded values indicate agreement between the evaluators.
FOIS, functional oral intake scale; TPN, total parenteral nutrition.
[a]TPN was performed without tube or oral feeding at the time of the examination. Rater 2 misinterpreted the meaning of TPN and classified it as FOIS level 2.

Among adult stroke patients, Crary et al. reported a high inter-rater reliability of the FOIS (absolute agreement, 85%) (7), and McMicken et al. reported ICC values of 0.975 and 0.964 at the time of admission and discharge, respectively (9). In the present study, the FOIS for children was associated with aspiration and dysphagia severity identified from the VFSS. This was similar to the results of a previous study, which evaluated the FOIS in stroke patients (7). Accordingly, the FOIS for infants may be appropriate for documenting feeding abilities and evaluating the effectiveness of interventions.

Implication of the Distinction Between FOIS Levels 2 and 3 in Infants

Both FOIS levels 2 and 3 could be categorized as concurrent tube and oral feeding, and our observers reported three disagreements between these levels when evaluating patients in our study (**Table 5**). One patient was an 11-month-old infant with myotonic dystrophy who received a total tube feeding volume of 700 cc per day in five or six doses, as well as 20–40 g of puree once per day. One evaluator regarded once-daily feeding as a consistent oral intake (i.e., FOIS level 3), whereas the other considered it a minimal attempt at oral intake (i.e., FOIS level 2). The second patient was a 9-month-old infant with CHARGE syndrome who received a total tube feeding volume of 700 cc per day in four or five doses and attempted to consume minimal amounts of puree orally with every meal. The last patient was a 7-month-old infant with Pierre–Robin syndrome who received a total tube feeding volume of 800 cc per day in six or seven doses, together with a soft blended oral diet (∼40 cc per day) at least once per day.

In our study, infants with POF who received a higher nutritional contribution from oral feeding were more likely to achieve FOF. This suggests that both the oral feeding amount and consistency should be considered when distinguishing FOIS levels 2 and 3. For example, eight out of 14 infants with <10% POF achieved FOF after 1 year, whereas 18 out of 19 infants with ≥10% POF achieved FOF after 1 year. Based on these results, we have revised the criteria for distinguishing FOIS levels 2 and 3 to consider both the oral intake amount and consistency, as shown in **Table 6**.

Implication of the Distinction Between FOIS Levels 4 and 5 in Infants

According to Pridham et al. infants can begin to consume semisolid food from a spoon between 5 and 7 months of age and complete this type of consumption at approximately 8 months of age (28). A 2001 guideline from the World Health Organization recommended the initiation of complementary feeding at 6 months of age and a concurrent and gradual solidification of foods (11). According to this guideline, the consumption of pureed, mashed, and semi-solid foods generally begins at 6 months of age, followed by the consumption of finger foods at 8 months and an adult-like diet at 12 months (11). In 2017, the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition Committee on Nutrition in 2017 recommended that complementary foods should be introduced between 4 and 6 months of age (29). From the neurodevelopmental point of view, lumpy (semisolid) food can be consumed between 6 and 12 months, and after 9 months, most infants can eat finger food and are able to chew their food (29). Northstone et al. reported a tendency toward feeding difficulties and the avoidance of certain foods if solid foods are not introduced until 9–10 months of age (12). Consistent with those studies, we defined the normal expansion of oral diet as the introduction of pureed foods before 9 months of age and of mashed foods and soft lumps before 12 months of age.

Among the 77 infants with FOF at the time of the VFSS in our study, the two raters reported five disagreements between FOIS levels 4 and 5. One such infant was assessed at 270 days of age and was consuming bottled milk. One examiner considered 270 days to be older than 9 months and evaluated the infant at FOIS level 4, whereas the other rater considered the infant younger than 9 months and evaluated him at FOIS level 5. Another infant was mainly consuming bottled milk at 305 days of age and had been attempting a 50-cc volume of pureed food 1 week before the evaluation. In this case, one examiner rated the feeding status as FOIS level 5 because she considered a pureed diet to be normal, whereas the other examiner assigned a rating of FOIS level 4 because the pureed diet had been initiated after 9 months of age. To improve the inter-rater reliability, it could be recommended to give a clear instruction that the FOIS for infants is based on the diet at the time of the evaluation.

STUDY LIMITATIONS

In this study, we were unable to evaluate the correlation between the FOIS for infants and developmental assessments. Future studies could potentially apply the Bayley Scales of Infant and Toddler Development (30) in conjunction with the FOIS assessment. Additionally, this was a single-center study, which may have led to selection bias. Moreover, the validity and reliability of the FOIS for infants were assessed with a heterogeneous disease group. Crary et al. (7) originally suggested the adult FOIS for stroke patients. Afterwards, other researchers expanded the FOIS for patients with traumatic brain injury (31, 32), head and neck cancer (33, 34), vocal fold immobility (35), vagal schwannoma resection (36), cerebral palsy

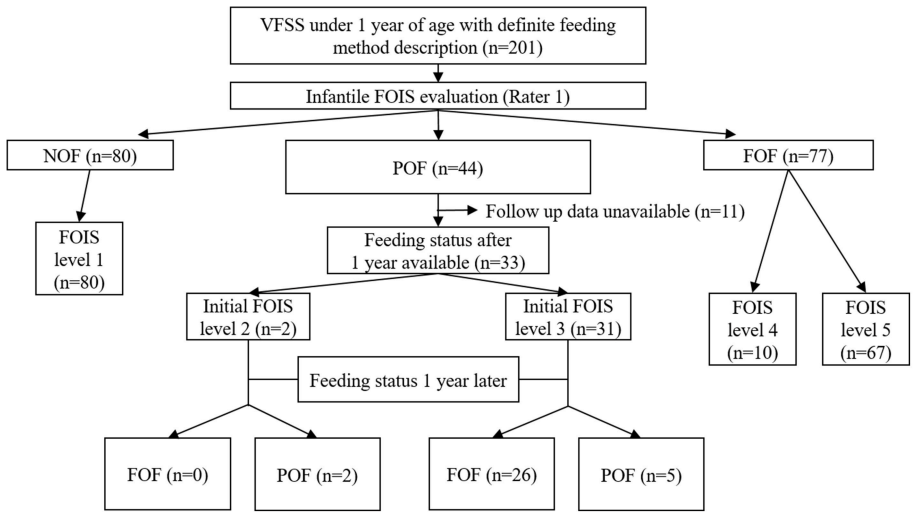

FIGURE 1 | Acquisition of data relevant to the FOIS for infants and the feeding status after 1 year FOIS, functional oral intake scale; VFSS, videofluoroscopic swallowing study; NOF, non-oral feeding; POF, partial oral feeding; FOF, full oral feeding.

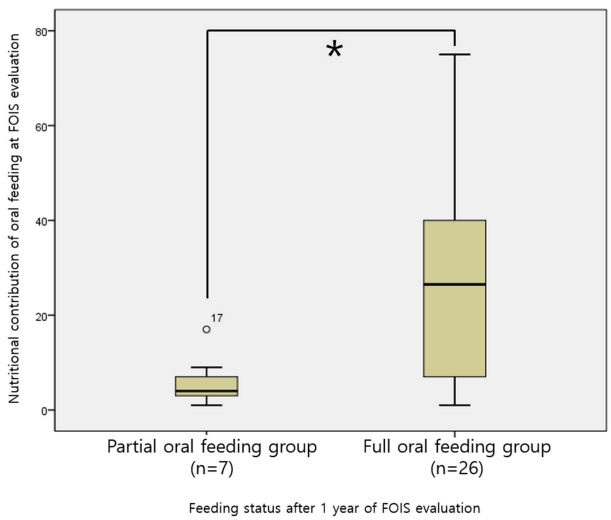

FIGURE 2 | Comparison of the caloric contributions of oral intake among POF infants stratified according to the achievement or non-achievement of FOF after 1 year *$p < 0.001$. POF, partial oral feeding; FOF, full oral feeding.

TABLE 6 | The functional oral intake scale for infants considering both attempts and amounts of oral intake at level 2.

	Intake
Level 1	Nothing by mouth
Level 2	Tube-dependent with minimal oral intake[a]
Level 3	Tube and oral feeding in parallel[b]
Level 4	Expansion of oral diet not reached[c]
Level 5	Expansion of oral diet reached[c]

[a] *"Minimal oral intake" indicates minimal attempts of or a very small amount of oral intake.*
[b] *"In parallel" indicates consistent oral intake with significant caloric contribution.*
[c] *Normal expansion of oral diet is defined as the introduction of solid foods in pureed form before 9 months of age and the introduction of mashed foods and soft lumps before 12 months of age.*

(37), postsurgical dysphagia (38), neurodegenerative diseases (39), postextubation dysphagia in children (40), and neurogenic dysphagia (41). The FOIS for infants suggested in the present study was a simplified scale with levels reduced from 7 to 5, without taking into account the concepts of single/multiple consistency food, special preparation or compensation, and food restriction. Therefore, the variation in applicability according to disease groups might be less than that in the adult population. However, the scale proposed in this study might be more appropriate for certain disease groups than other groups, and in some groups, this scale would not be applicable. For example, the FOIS suggested in this study could be inappropriate for infants who require continuous total parenteral nutrition because of gastrointestinal problems.

CONCLUSIONS

The FOIS for infants, which reflects the expansion of oral diet in infants, showed adequate reliability and validity. Our findings suggest that this scale could be useful for documenting infants' feeding abilities and evaluating the effectiveness of interventions. The reliability and validity of the FOIS for infants could be improved if caloric contribution as well as the consistency of oral feeding are considered for the distinction between FOIS levels 2 and 3.

ETHICS STATEMENT

All study-related procedures were performed in accordance with the ethical standards of the institutional and/or national research committee and the 1964 Declaration of Helsinki. Ethical approval for the study was obtained from the Seoul National University Hospital Institutional Review Board

(IRB) (No. 1807-189-963), which waived the requirement for informed consent due to the retrospective nature of the study.

AUTHOR CONTRIBUTIONS

YY: acquisition of data, analysis and interpretation of data, writing and critical revision of manuscript. H-IS: study concept and design, acquisition of data, analysis and interpretation of data, study supervision, and critical revision of manuscript for intellectual content.

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Early Inflammatory Status Related to Pediatric Obesity

Cristina Oana Mărginean[1], *Lorena Elena Meliţ*[1]*, *Dana Valentina Ghiga*[2] and *Maria Oana Mărginean*[3]

[1] Department of Pediatrics, University of Medicine, Pharmacy, Sciences, and Technology, Târgu Mureş, Romania, [2] Department of Medical Informatics and Biostatistics, University of Medicine, Pharmacy, Sciences and Technology, Târgu Mureş, Romania, [3] Department of Pediatric Cardiology, University of Medicine, Pharmacy, Sciences and Technology, Târgu Mureş, Romania

Background: Obese individuals are often in a chronic inflammatory condition due to the malfunction of immune-related activities in the adipose tissue, involving a transient infiltration of neutrophils within the abdominal fat and their binding to adipocytes. Neutrophil to lymphocyte ratio (NLR) and platelet to lymphocyte ratio (PLR) are considered cost-effective markers for the detection of subclinical inflammation. Our study intends to assess the early stages of inflammation associated with overweight and obesity in children.

Materials and Methods: We performed a prospective study with 164 children, aged between 5 and 18 years, admitted to a Pediatric Tertiary Hospital in Romania between January 2018 and January 2019. The patients were divided according to body mass index (BMI) into two groups: Group 1: 77 overweight and obese children (BMI percentile $\geq$85), and Group 2: 87 children with a normal BMI, in order to evaluate the correlation between BMI and laboratory parameters (CBC, ESR, transaminase, total protein, albumin, and blood glucose levels), inflammatory biomarkers, NLR and PLR, and changes in abdominal ultrasound findings.

Results: We found that the leukocyte, lymphocyte, erythrocyte, platelet, CRP, and transaminase levels were significantly higher in the overweight/obese group ($p = 0.0379$, $p = 0.0002$, $p = 0.0003$, $p = 0.0006$, $p < 0.0001$, $p = 0.0332$, and $p < 0.0001$, respectively). No significant statistical differences between the two groups in terms of neutrophil, hemoglobin, albumin, total protein, and glycemia levels were noted ($p > 0.05$). Moreover, NLR and PLR did not differ significantly between the two groups ($p = 0.4674$ and $p = 0.9973$, respectively).

Conclusions: Obesity is associated with systemic low-grade inflammation which is reaching alarming rates worldwide among both children and adults. Our study proved that leukocyte, lymphocyte, erythrocyte, and platelet levels are significantly higher in overweight/obese children, emphasizing the inflammatory status related to this condition. Therefore, obesity-related studies involving pediatric patients are of major interest in order to develop appropriate methods to prevent the development of further complications in adulthood.

Keywords: children, overweight, obese, neutrophil to lymphocyte ratio (NLR), platelet to lymphocyte ration (PLR)

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King's College London,
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Instituto Nacional de Salud del Niño, Peru

***Correspondence:**
Lorena Elena Meliţ
lory_chimista89@yahoo.com

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INTRODUCTION

Obesity is a current global health problem in both children and adults and causes a significant burden. Most studies investigating obesity involves adults; however, it is essential for studies to focus on childhood obesity in order to prevent its associated complications and further development in adulthood. The incidence of this nutritional disorder in children has reached alarming rates worldwide. In Romania, one in four children were found to be either overweight or obese (1). Its etiology is complex, involving an interaction between genetic susceptibility and environmental or "obesogenic" factors, which play a key role in triggering obesity, representing therefore the basis for successful interventions (2).

It is well-documented that obese individuals express a chronic inflammatory status; obesity-related complications, such as cardiovascular disease, type 2 diabetes mellitus, metabolic syndrome, and non-alcoholic steatohepatitis were proven to be results of obesity-associated low-grade inflammation (3, 4). This obesity-related inflammation is due to the malfunction of immune-related activities in the adipose tissue, involving a transient infiltration of neutrophils within the abdominal fat and their binding to adipocytes (5). This process may precede macrophage infiltration similar to that in other inflammatory conditions (5, 6). Moreover, neutrophils are the most important and abundant subtype of white blood cells in human peripheral blood. Neutrophils and chronic inflammation seem to be linked to chronic hypertension and obesity (7), with the total count of circulating neutrophils being increased in obese individuals (8). Besides neutrophils, leukocytes are also associated with obesity-induced chronic inflammatory status, being equally involved in related comorbidity development. Based on this premise, obese individuals without comorbidities are considered to represent a special subgroup in the early stage of inflammation similar to overweight individuals (9).

Multiple serum markers are said to be associated with low-grade chronic inflammation. Neutrophil to lymphocyte ratio (NLR) is a recently discovered, cost-effective marker for the detection of subclinical inflammation that correlates with C-reactive protein (CRP) levels (10–12). This useful marker has been related to multiple inflammatory conditions, cardiovascular diseases, and cancer (13, 14). NLR was shown to be directly related to the degree of inflammation (15). Platelet to lymphocyte ration (PLR) is another biomarker that can be calculated based on the complete blood count (CBC) and has been proven to be useful in the diagnosis and monitoring of several systemic inflammatory processes (16–19). PLR is an indicator of the balance between inflammation and thrombosis. Thus, the inflammatory status results in accelerated megakaryocyte proliferation and associated thrombocytosis. Moreover, increased platelet counts and decreased lymphocyte counts have been shown to be related to both aggregation and inflammation, and thus, represent risk indicators (20, 21). Other blood parameters, such as lymphocytes, monocytes, hemoglobin, erythrocyte sedimentation rate (ESR), total proteins, albumin, iron, cholesterol, triglycerides, and transaminases, and different gene polymorphisms, are related to childhood obesity and overweight (22–29).

The aim of this study was to assess different blood parameters associated with the low grade inflammatory status in overweight and obese children in order to detect the early stages of inflammation related to this nutritional disorder.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CBC, complete blood cellular; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; n, number; NLR, neutrophil/lymphocyte ratio; PLR, platelets/lymphocyte ratio; SD, standard deviation.

MATERIALS AND METHODS

Study Sample

We performed a prospective study with 191 pediatric patients aged between 5 and 18 years, referred to a Pediatric Tertiary Hospital in Romania, between January 2018 and January 2019 that were assessed on a 1 day admission chart system, without requiring long time hospitalization. However, the parents of only 173 children agreed to sign the informed consent form for their children to be included in the study. After selection according to age and sex, only 164 children were finally included in the study. The children were divided according the value of body mass index (BMI) into two groups: group 1, the study group: 77 overweight and obese children (overweight children: BMI percentile $\geq$85 and $<$95, and obese children: BMI percentile $>$95), and group 2, the control group: 87 children with normal BMI (percentile $\geq$5 and $<$85). The inclusion criteria for both groups was an age of between 5 and 18 years. In the study group we included children with a BMI percentile $>$85, while in the control group we included those with a BMI percentile $\geq$5 and $<$85 (30–32). The exclusion criteria consisted of age below 5 years, secondary obesity, patients with obesity-related complications, chronic disorders, infectious diseases, incomplete data, and patients whose parents refused to sign the informed consent form.

All patients underwent a thorough anamnesis and clinical exam; blood parameters were also measured: CBC, ESR, CRP, transaminase, total protein, albumin, and blood glucose levels. Inflammatory biomarkers, such as NLR and PLR, were calculated by dividing the neutrophil count and platelet count, respectively, by the lymphocyte count. The abdominal ultrasound was performed in all children by a single trained clinician. The laboratory parameters were assessed using a Cobas Integra 400 plus automated analyzer, Roche Diagnostics GmbH, Mannheim, Germany. The ultrasound exams were performed with a probe, with variable frequencies between 2.5 and 6 MHz, and an S8 General Electric Machine.

All parents/caregivers signed the informed consent for their children. The study was approved by the Ethics Committee of the University of Medicine and Pharmacy of Târgu Mureş (No 329/November 17th 2017), and it was performed according to the principles of the Helsinki Declaration.

Statistical Analysis

The children' characteristics are presented as means $\pm$ standard deviation and medians.

TABLE 1 | The descriptive analysis of the blood parameters in the two groups.

Laboratory parameters	Study group(n = 77) Mean ± SD	Control group (n = 87) Mean ± SD	p-value
Age (years)	10.79 ± 3.503	12.33 ± 3.516	*0.0052
Birth weight (kg)	3.310 ± 0.5603	3.278 ± 1.070	*0.1400
Current weight (kg)	57.78 ± 20.94	45.36 ± 14.46	**<0.0001**
Height (cm)	146.8 ± 17.83	152.7 ± 16.19	0.0263
BMI (kg/m^2)	25.82 ± 4.518	18.92 ± 2.934	**<0.0001**
Leukocytes (10^3/μl)	7.932 ± 2.885	7.162 ± 1.896	***0.0379**
Neutrophils (10^3/μl)	3.848 ± 2293	3.309 ± 2210	*0.1063
Lymphocytes (10^3/μl)	2871 ± 1042	2382 ± 743.3	***0.0002**
Hemoglobin (g/dl)	13.55 ± 1.277	13.45 ± 1.193	*0.1776
Erythrocytes (10^6/L)	4.989 ± 0.3986	4.811 ± 0.3863	***0.0003**
Platelets (10^3/μl)	338.0 ± 100.3	290.0 ± 66.27	**0.0006**
Albumin (g/dL)	4.892 ± 0.3255	4.831 ± 0.3290	0.2295
Total proteins (g/dL)	7.511 ± 0.4958	7.470 ± 0.4247	0.5676
AST (UI)	26.87 ± 23.78	22.37 ± 12.18	***0.0332**
ALT (UI)	27.17 ± 43.14	14.68 ± 9.655	***<0.0001**
Glycaemia (mg/dl)	86.33 ± 9.527	84.87 ± 9.171	*0.3338
NLR	1.577 ± 0.8019	1.878 ± 1.527	*0.4673
PLR	125.9 ± 44.77	131.5 ± 47.21	*0.9973
ESR	12.55 ± 7.965	11.32 ± 8.690	*0.2122
CRP (mg/l)	4.508 ± 2.839	1.938 ± 4.729	***<0.0001**

*ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CRP, C-reactive protein; NLR, neutrophil/ lymphocyte ratio; ESR, erythrocyte sedimentation rate; n, number; PLR, platelets/lymphocyte ratio; SD, standard deviation; *Mann-Whitney test was used. n = number. Bold represents the statistically significant values.*

The statistical analysis comprised of descriptive statistical analysis (frequency, media, median, and standard deviations) and inferential statistical elements. D'Agostino & Pearson's tests were applied in order to identify the distribution of the series of analyzed data. We applied the Student t-test for unpaired data, and the Mann-Whitney test for comparison of the medians. A Chi squared test was used for association determination. The significance threshold was considered at a p-value of 0.05. The statistical analysis was performed using GraphPad Prism 7 free trial version, GraphPad Software Inc., California, USA.

RESULTS

Among the 164 children included in the study, the mean age for the study group was 10.79 ± 3.503 years, whereas that for the control group was 12.33 ± 3.516 years ($p = 0.0052$). Regarding residence area, we found the same distribution of obese children among rural and urban areas ($p = 0.8338$), without a significance influence of the social status in the development of obesity. According to sex distribution, being overweight/obese was more common in boys ($p = 0.0083$). Thus, our sample is age- and sex-matched. We also assessed the birth weight, identifying a higher value for control group, 3.278 ± 1.070 kg in comparison to 3.310 ± 0.5603 kg for obese children, but without statistical significance ($p = 0.1400$). The current weight and BMI of the children included in our study were significantly higher for obese group ($p < 0.0001$).Thus, the mean value of the current weight was 57.78 ± 20.94 kg for obese children versus 45.36 ± 14.46 kg for control group, while the mean value of BMI in case of obese children was of 25.82 ± 4.518 kg/m^2 compared to 18.92 ± 2.934 kg/m^2 for normal weight children. Contrariwise, the height was higher for control group, though without statistical significance ($p = 0.0263$), with a mean value of 152.7 ± 16.19 cm for control group in comparison to 146.8 ± 17.83 cm for obese children. All these descriptive parameters along with the values of the laboratory parameters are mentioned in **Table 1**.

Among the blood parameters, we found that leukocyte (**Figure 1**), lymphocyte (**Figure 2**), erythrocyte, platelet (**Figure 3**), CRP (**Figure 4**), and transaminase levels (AST and ALT) were significantly higher in the overweight/obese children in contrast to the control group ($p = 0.0379$, $p = 0.0002$, $p = 0.0003$, $p = 0.0006$, $p < 0.0001$, $p = 0.0332$, and $p < 0.0001$, respectively).

Nevertheless, we did not encounter any significant statistical differences between the two groups in terms of neutrophil, hemoglobin, albumin, total protein, erythrocyte sedimentation rate (ESR), and glycemia levels ($p = 0.1063$, $p = 0.1776$, $p = 0.2295$, $p = 0.5676$, $p = 0.2122$, and $p = 0.3338$, respectively). Moreover, NLR and PLR did not differ significantly between the two groups ($p = 0.4674$ and $p = 0.9973$, respectively).

Regarding the results of the abdominal ultrasound, we found pathological changes, such as hepatomegaly and hepatic steatosis, in 77.92% of the overweight/obese children, whereas, in the control group, only three children were identified to have hepatomegaly (3.44%). Thus, hepatomegaly and hepatic steatosis were significantly more frequent in the overweight/obese group than in the normal BMI group ($p > 0.0001$) (**Table 2**).

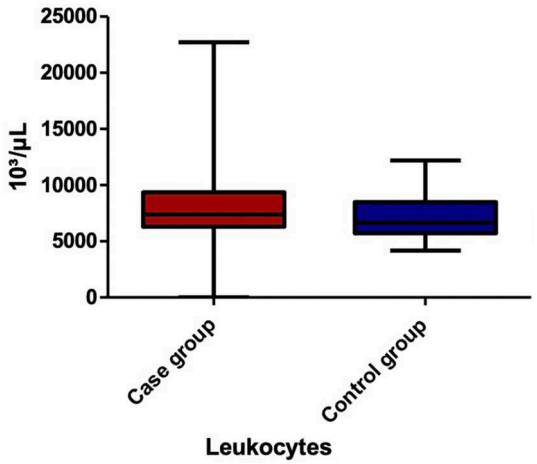

FIGURE 1 | The comparison of leukocyte count between the two groups.

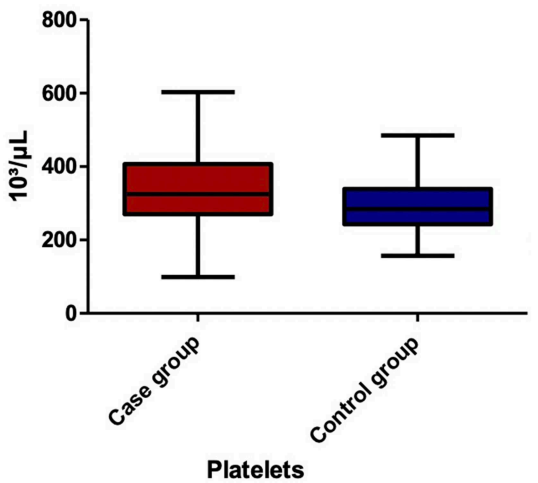

FIGURE 3 | The comparison of platelet count between the two groups.

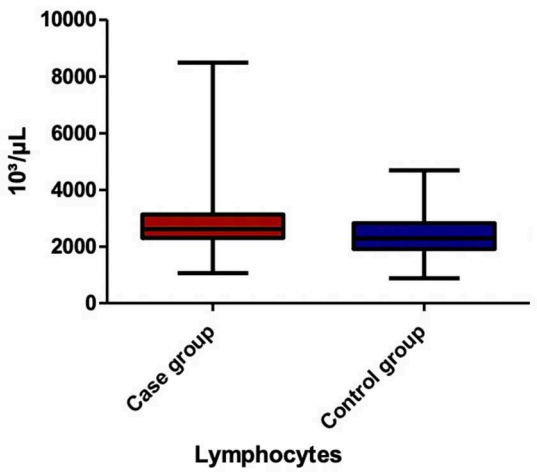

FIGURE 2 | The comparison of lymphocyte count between the two groups.

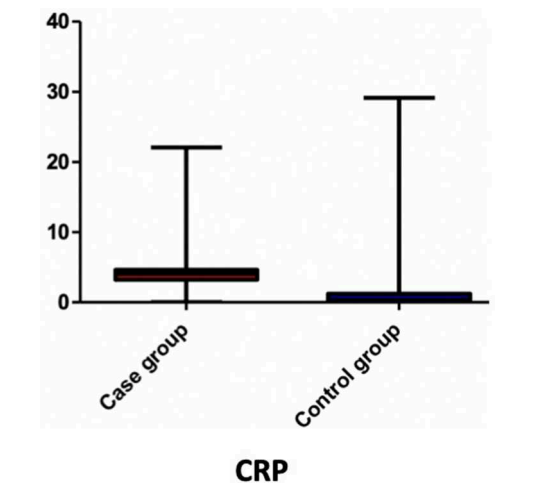

FIGURE 4 | The comparison of CRP value between the two groups.

DISCUSSIONS

The World Health Organization stated that the prevalence of obesity worldwide has nearly tripled since 1975 (33). Thus, in 2016, 41 million children below the age of 5 years were overweight or obese (33). The prevalence of obesity increases with age, with over 340 million children and adolescents aged between 5 and 19 years being affected with this condition in 2016 (33). Obesity is an important leading cause of mortality worldwide, being associated with increased risk for heart failure, stroke, skeletal system diseases, and malignancies (33).

The adipose tissue has been proven to contribute to both the initiation and maintenance of systemic inflammation (5, 34). Adipose tissue-related inflammation leads to a wide variety of immune responses, involving neutrophil participation in the early phases followed by macrophage involvement and mast cell polarization (35, 36). Studies performed on mice proved that the neutrophil count significantly increases within the adipose tissue even in the first days after the initiation of a high fat diet (37), suggesting that obesity, even in its early stages, is associated with systemic inflammation. Similar findings were also reported in humans in the study of Tam et al. (38), who underlined that acute lipid overload is related to the increase of inflammatory biomarkers, such as CRP and monocyte chemoattractant protein-1. Our study sustains the findings of Tam et al. regarding the CRP, which was significantly higher in the obese/overweight children included in this research. Recently, a wide range of biomarkers were found to be related to low-grade systemic inflammations, such as CBC parameters, NLR, PLR, mean platelet volume, and CRP among others. Thus, it was proven that the peripheral blood of otherwise healthy overweight/obese individuals exhibit characteristics of a low-grade inflammation (6).

TABLE 2 | Comparative analysis of liver ultrasound findings between the two groups.

Abdominal ultrasound	Study group (77 cases)	Control group (87 cases)
Hepatomegaly	13	3
Normal	17	84
Steatosis	19	0
Hepatomegaly, steatosis	27	0
Steatohepatitis areas surrounding the gallbladder	1	0

White blood cells were shown to be associated with the development of metabolic syndrome (39, 40). A study performed with 6,700 patients found positive correlations between waist circumference and leukocyte, lymphocyte, neutrophil, and platelet levels, and medium platelet volume (41). Moreover, another study including 223 subjects showed that an increase in BMI results in increased leukocyte, lymphocyte, platelet, and neutrophil counts (42). Similar to the findings of the previously mentioned study, our study showed a significant correlation between being overweight/obese and leukocyte, platelet, lymphocyte, and erythrocyte counts. However, it failed to show a significant association of these conditions with the neutrophil count. Despite the fact that NLR and PLR are immune response markers related to chronic inflammation (19, 43), we did not find significant correlations between these markers and obesity/overweight. The lack of correlation between NLR and BMI in our study might be explained by the significant increase in lymphocyte count among overweight/obese children. Most likely, PLR did not correlate with an increased BMI in our study due to the significant increase in both platelet and lymphocyte counts in the overweight/obese group. Recent studies emphasize the role of NLR as a potential inflammation marker in not only cardiac and non-cardiac disorders, but also autoimmune conditions and infections (44, 45). Inflammatory status is reflected by neutrophil counts, while the lymphocyte count is linked to the nutritional status and general stress (46). The increase in neutrophil count was proven to be directly related to the degree of obesity (47). Thus, the fact that in our study we did not find a significant increase in neutrophil count among the study group might be explained by the young age of our subjects, resulting in an insufficient amount of time for chronic inflammation to occur. Moreover, multiple studies have focused on assessing the role of NLR and PLR in patients with related-comorbidities; a study performed with 155 obese and non-obese patients with obstructive sleep apnea found significantly higher values in terms of NLR among obese individuals (46). Metabolic syndrome related obesity is another disorder commonly associated with chronic inflammation that was found to be related with NLR (48). Nevertheless, the findings remain contradictory because similar to our study, Bahadir et al. also failed to prove an association between NLR and obesity or metabolic syndrome (49). Diabetes mellitus and its related metabolic malfunctions are also associated with an increased NLR (50).

Most obesity-related studies have been performed using adult participants and stated that the wide range of associated comorbidities hinder the elucidation of a cause-effect link between inflammation and obesity (51). Similar to our study, Aydin et al. performed a study with 187 children (130 obese individuals and 57 healthy controls) (51). They found that the age of obese children was lower than that of the healthy controls in their study. Regarding blood parameters, the authors showed that lymphocyte and neutrophil counts, and NLR were significantly higher in obese children. Similarly, our study also showed a significant increase in lymphocyte count among overweight/obese children, but it failed to show significant differences in terms of neutrophil count and NLR between the normal BMI children and those with high BMI. Moreover, the previously mentioned study (51) did not identify any significant differences between obese and normal weight children with respect to PLR, as in our study. These contradictory findings might be explained by the fact that pediatric cases are rarely accompanied by obesity-related complications.

The limitations of our study consist of the relatively small sample size and the lack of correlation with dietary habits and the assessment of related complications. Conversely, this study will make a significant contribution to the existing literature as it involved pediatric patients and was able to identify the risk factors associated with the early phases of obesity-related inflammatory process. Moreover, to the best of our knowledge, this is the first study in Romania that assessed the role of CBC parameters, NLR, and PLR in overweight/obese children.

CONCLUSIONS

Obesity is associated with systemic low-grade inflammation and is reaching alarming rates worldwide among both children and adults. Our study proved that leukocyte, lymphocyte, erythrocyte, and platelet counts are significantly higher in overweight/obese children, emphasizing the early inflammatory status related to this condition. Therefore, obesity-related studies involving pediatric patients are crucial in order to develop appropriate methods for preventing the development of further complications in adulthood.

ETHICS STATEMENT

The study was approved by the Ethics Committee of the University of Medicine and Pharmacy of Târgu Mureş (No 329/ November 17th 2017), and it was performed according to the principles of the Helsinki Declaration. The consent procedure used in our study consisted in an informed written consent that was signed by all parents/caregivers for their children prior to

the inclusion in the study. The study was also explained to the children signed and we obtained their verbal assent.

AUTHOR CONTRIBUTIONS

CM, LM, and MM conceptualized and designed the study, drafted the initial manuscript, and reviewed, and revised the manuscript. MM and LM designed the data collection instruments, collected data, carried out the initial analyses, and reviewed and revised the manuscript. DG performed the statistical analysis. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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CD4$^+$T Cell Subset Profiling in Biliary Atresia Reveals ICOS$^-$ Regulatory T Cells as a Favorable Prognostic Factor

Shuhao Zhang[1†], Shyamal Goswami[2†], Jiaqiang Ma[2†], Lu Meng[2], Youping Wang[2], Fangming Zhu[2], Dandan Zhang[3], Shan Zheng[1], Rui Dong[1], Xianmin Xiao[1], Xiaoming Zhang[2] and Gong Chen[1*]*

[1] Department of Pediatric Surgery, Children's Hospital of Fudan University, Shanghai, China, [2] Key Laboratory of Molecular Virology and Immunology, Institut Pasteur of Shanghai, Chinese Academy of Sciences, Shanghai, China, [3] MOE Key Laboratory of Metabolism and Molecular Medicine, Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Fudan University, Shanghai, China

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Jan B. F. Hulscher,
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School of Medicine, University of
Zagreb, Croatia

***Correspondence:**
Xiaoming Zhang
xmzhang@ips.ac.cn
Gong Chen
chengongzlp@hotmail.com

[†]These authors have contributed equally to this work

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Biliary atresia (BA) is a destructive pediatric liver disease and CD4$^+$T cell activation is demonstrated to play an important role in BA. However, a comprehensive scenario regarding the involvement of CD4$^+$T cell subsets to the development of BA remains unclear. Here, we aim to explore the infiltration of CD4$^+$T cell subsets and their clinical significance in BA. In the present study, thirty BA liver samples were collected during surgery and were divided into good (BA1, $n = 16$) and poor prognosis (BA2, $n = 14$), with samples from choledochal cyst patients ($n = 8$) as control. By using multiplex immunohistochemistry, we evaluated the infiltration level of CD4$^+$T cell subsets in the portal areas. RT-qPCR and flow cytometry were further applied to explore detailed features of Treg subsets. We revealed that hepatic infiltrating Th1, Th2, Th17, and ICOS$^+$Treg cells were significantly increased in BA patients compared to controls and were negatively associated with prognosis, while high infiltrating ICOS$^-$Tregs showed a favorable outcome. Phenotypic analysis indicated that, in contrast to ICOS$^+$Tregs, ICOS$^-$Tregs were mainly CD45RA^{hi}CD45RO^{low}, and preferentially expressed more CD73. Besides, RT-qPCR revealed elevated expression of *CD25*, *CD73*, *TGF-β,* and *BCL-2* genes in ICOS$^-$Tregs. Finally, functional assay confirmed that ICOS$^-$Tregs had a higher suppressive capacity to cytokine secretion and were more resistant to apoptosis *in vitro*. Collectively, we demonstrate that a mixed immune response is involved in BA pathogenesis, and the globally enhanced effector CD4$^+$T cell response is associated with unfavorable prognosis, highly suppressive ICOS$^-$Tregs is a protective factor and may serve an important reference to predict prognosis.

Keywords: biliary atresia, immune dysfunction, CD4$^+$T cell subset, inducible co-stimulator, prognosis

INTRODUCTION

Biliary atresia (BA) is a childhood disease characterized by fibroinflammatory obstruction of the extrahepatic and intrahepatic bile ducts. BA development is always associated with persistent and progressive inflammatory response resulting in progressive jaundice and rapid fibrosis (1). During inflammation, the portal area is infiltrated with characteristic inflammatory cells, consisting of $CD4^+$T cells, $CD8^+$T cells, and Kupffer cells (2–4). Dendritic cells (5) and natural killer cells (6) could injure the biliary epithelium, and T cells further induce bile duct obstruction, liver fibrosis, and cirrhosis by excessive production of cytokines such as IFN-γ, IL-6, and IL-17. However, the exact role of T cells in pathogenesis of BA remains obscure.

Effector T cells induced autoimmune attack is one of the important causes of bile duct injury. While, the immunosuppressive regulatory T (Tregs) cells have been shown to exhibit a protective role in BA (7, 8). Tregs are not a homogenous population, but can be divided into two subsets based on ICOS expression. $ICOS^+$Tregs preferentially secrete high amounts of IL-10 and moderate levels of TGF-β1, while $ICOS^-$Tregs exert a suppressive function primarily through secreting TGF-β1 (9). Therefore, the two Treg subsets with different functions can play opposite roles in some diseases, such as hepatocellular carcinoma (10) and melanoma (11). The functionality and co-existence of these two Treg subsets in BA is still unknown.

Previous studies mainly focused on single immune cell type in BA, while a comprehensive picture of the major $CD4^+$T cell subsets is lacking. In addition, most of previous reports had neglected the *in-situ* contextual link between immune cell type infiltration and disease outcome. With the aid of our recently-developed multiplex immunohistochemistry (mIHC) technique, we could further explore the link between the infiltration of these $CD4^+$T cell subsets (Th1, Th2, Th17, and Tregs) in the liver tissues and disease outcomes in BA patients. Furthermore, extended investigations with flow cytometry and functional assays were also performed to study the $ICOS^+$ and $ICOS^-$Tregs.

MATERIALS AND METHODS

Patients

Patients for Immunohistochemistry

The histopathological liver sections of 30 BA patients who underwent Kasai portoenterostomy (KPE) at Children's Hospital of Fudan University (Shanghai, China) were collected within the period of February 2015 to March 2017. Postoperative serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), direct bilirubin (DB), total bilirubin (TB) levels are reliable indexes for predicting the prognosis of BA patients (12–15). Thus, patients were divided into two groups based on these indexes 6 months after surgery. Clinicopathologic features of the 30 patients were provided in **Table 1**. Poor prognosis (BA2 group, $n = 14$) was defined as the serum TB level more than 17.1 μmol/L, serum DB level more than 6.8 μmol/L and the liver enzymes were abnormal (ALT > 50 U/L, AST > 50 U/L). The rest of the patients were good prognosis (BA1 group, $n = 16$). Eight liver sections from choledochal cysts were considered as healthy control (clinicopathologic features were summarized in **Table 1**).

Patients for Flow Cytometry and Functional Assays

Peripheral blood samples and freshly resected liver tissues from 24 BA patients were harvested during KPE at Children's Hospital of Fudan University (Shanghai, China) in June 2017. Five of them were used to detect the ICOS expression on Tregs; five of them were used for phenotypic analysis; four of them were used for RNA isolation; four of them were used for apoptosis experiment; six of them were used for coculture experiment. Peripheral blood samples from 10 patients, including brachial plexus injury ($n = 4$) and accessory ear ($n = 6$) were included as the control.

Multiplex Immunohistochemistry

Multispectral imaging was performed as described in the supplementary section of Feng et al. (16) with appropriate optimization. The liver sections were deparaffinized in three changes of xylene and two changes of 100% ethanol and subsequent gradation of 95, 80, and 70% alcohol for 3 min each. After being heat-induced epitope retrieval with a preheated epitope retrieval solution (pH 8.0, Enzo Life Sciences, Inc. USA), endogenous peroxidase was inactivated by incubation in 3% H_2O_2 for 20 min. Next, the sections were pre-incubated with 10% normal goat serum and then incubated overnight with primary antibodies: CD4, Foxp3, T-bet, GATA3, ICOS, and RORγt (details in **Table S1**). The next day, sections were incubated with the HRP-conjugated second antibody (Vector) for 20 min at room temperature. After washing, polymer tagged HRP mediate the covalent binding of a different fluorophore (Opal-520, Opal-570, Opal-620, Opal-650, and Opal-690) sequentially, coupled with tyramide signal amplification (TSA) step as specified by the manufacturer (Perkin Elmer Inc.). At last, sections were counterstained with DAPI (Sigma-Aldrich). Slides were imaged using the PerkinElmer Vectra platform and a 0.3345 mm^2 area containing at least one portal area was analyzed in batches using PerkinElmer inForm® software and cell quantification of positively stained cells was analyzed by R script.

Flow Cytometry and Apoptosis Assay

Peripheral Blood Mononuclear Cells (PBMCs) were isolated by Ficoll density gradient (Amersham, Uppsala, Sweden). Then single cell suspensions were stained with fluorochrome-conjugated antibodies against CD4, CD25, CD127, ICOS, CD39, CD73, CD45RO, CD45RA, and PE-mouse Isotype (details in **Table S2**) to identify the phenotypes of Treg subsets. Gating on Tregs was same as the sorting gates (**Figure S1**). Data were acquired by BD LSRFortessa. APC-Annexin-V (Cat:640941 Biolegend) and PI (Sigma) were used to assess cell apoptosis.

Abbreviations: BA, biliary atresia; KPE, Kasai portoenterostomy; ICOS, inducible costimulator; AST, aspartate aminotransferase; ALT, alanine aminotransferase; TB, total bilirubin; DB, direct bilirubin; ADA, absence of adenosine deaminase; GGT, gamma-glutamyl transpeptidase.

TABLE 1 | Demographic and laboratory data of BA patients and control patients.

	Control ($n = 8$)	BA1 group ($n = 16$)	BA2 group ($n = 14$)	p-Value (BA1 vs. BA2)
PREOPERATIVE DATA				
Operative age(day)	992.9 ± 777.6	56.0 ± 11.3	56.9 ± 7.2	>0.05
Gender(F:M)	5:3	10:6	9:5	>0.05
Weight(kg)	13.1 ± 6.24	4.8 ± 0.5	4.6 ± 0.6	>0.05
PLT	237.3 ± 92.4	356.7 ± 194.8	328.6 ± 105.0	>0.05
GGT	—	675.3 ± 439.0	633.9 ± 471.6	>0.05
AST(U/L)	101.9 ± 137.8	245.9 ± 181.3	144.5 ± 89.5	>0.05
ALT(U/L)	88.1 ± 99.8	112.8 ± 75.4	102.1 ± 53.4	>0.05
TB(μmol/L)	85.3 ± 91.2	162.3 ± 43.5	145.5 ± 67.9	>0.05
DB(μmol/L)	55.1 ± 56.7	110.1 ± 33.4	99.6 ± 18.9	>0.05
SIX MONTHS AFTER SURGERY				
AST(U/L)	45.1 ± 52.7	41.6 ± 9.6	188.4 ± 156.3	<0.05
ALT(U/L)	38.5 ± 52.0	31.8 ± 17.3	168.7 ± 138.2	<0.05
TB(μmol/L)	14.1 ± 16.9	7.3 ± 2.5	90.9 ± 67.9	<0.05
DB(μmol/L)	8.5 ± 12.9	2.1 ± 0.9	72.1 ± 59.7	<0.05
Follow-up(month)	—	19.1 ± 5.8	8.6 ± 4.0	<0.05
AT SIXTH MONTH AFTER SURGERY				
TB(μmol/L)	—	7.6 ± 5.1	91.4 ± 69.6	<0.05
DB(μmol/L)	—	3.8 ± 4.1	73.5 ± 60.2	<0.05

Data were shown as the mean ± standard error (SEM).
M, male; F, female; PLT, Platelets; GGT, gamma-glutamyl transpeptidase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; DB, direct bilirubin; TB, total bilirubin; control, choledochal cyst; BA1, good prognosis; BA2, poor prognosis.

Isolation of $CD3^+CD25^-$T Cells, $ICOS^+$Tregs and $ICOS^-$Tregs

PBMCs were acquired as above. Then PBMCs were incubated with CD3 biotin (Biolegend, Clone. OKT3) and anti-biotin beads (Miltenyi Biotec) to acquire the $CD3^+$T cells by positive selection. $CD3^+$T cells were divided into two parts. One part was stained with PB-anti-SAV and CD25 to isolate $CD3^+CD25^-$T cells with BD FACSMelody. Another part was stained with CD4, CD25, CD127, and ICOS to isolate $ICOS^+$Tregs and $ICOS^-$Tregs. Sorting gates were shown in **Figure S1**.

RNA Isolation and Quantitative RT-PCR

Total cellular RNA was extracted from $ICOS^+$Tregs and $ICOS^-$Tregs of BA patients' peripheral blood with TRIzol reagent (Invitrogen, cat. 15596026/15596018) and subjected to reverse transcription with PrimeScript RT reagent Kit (Takara, cat. RR037A) according to the manufacturers' instructions. The expressions of *ICOS*, *CD25*, *CD39*, *CD73*, *TGF-β*, and *BCL-2* were analyzed via RT-qPCR with a SYBR Premix EX Taq (Tli RNaseH plus, Takara, cat. RR420A) with primers listed in **Table S3**. For analysis, expression levels of the genes were normalized to the values of beta-actin. Analysis of relative gene expression data using real-time quantitative PCR was calculated with the 2-ΔΔCt method (17).

Co-culture Experiment of Sorted $CD3^+CD25^-$T Cells and Treg Subsets

$CD3^+CD25^-$T cells were cultured alone or with $ICOS^+$Tregs and $ICOS^-$Tregs, respectively, in 96-well round-bottom plates in RPMI 1640 complete medium in the presence of 10 ng/ml human IL-2 and 0.5 μM 5′-AMP (Sigma-Aldrich) for 3 days at 37°C. Dynabeads Human T-Activator CD3/CD28 (ThermoFisher, Cat. 111.31D) was used to stimulate T cells and added at a ratio of 1:5 (beads: T responder). Treg subsets were added at a ratio of 1:2 (Treg subset: T responder). After 3 days, cells were restimulated with 50 ng/mL PMA and 1 μg/mL Ionomycin in the presence of GolgiPlug for 5 h. Dead cells were removed by the Live/Dead dye Zombie Yellow (Biolegend). Then, cells were stained with anti-CD4, CD8, IFN-γ, IL2, and TNFα antibodies (details in **Table S2**). Data were acquired by BD LSRFortessa.

Statistical Analysis

Data were shown as the medians ± IQRs or mean ± standard error (SEM) depending on data characteristics. Statistical analysis was performed with SPSS18.0 and Graphpad Prism 6. Statistical p-values were analyzed by a two-tailed Student's t-test. Correlation analyses were performed using Spearman's test. Survival curves were drawn by Kaplan–Meier univariate estimates and performed using classification as "low" or "high" according to the Youden index. Multivariate analysis was performed by Cox regression analysis. p-values < 0.05 were considered statistically significant.

RESULTS

Increased Infiltration of Th1, Th2, and Th17 Cells in the Portal Area of Livers From BA Patients

$CD4^+$ Th subsets had been implicated as important immune cells correlate to the pathogenesis of BA. As expected, Th

subset markers CD4, T-bet, GATA-3, and ROR-γt could be detected in the portal areas of BA livers (**Figures S2A–D**) and a more concrete picture of the infiltrated Th1 ($CD4^+$T-bet^+), Th2 ($CD4^+$GATA-3^+), and Th17 ($CD4^+$ROR-γt^+) *in situ* were shown in **Figures S3A–C** and **Figures 1A,B**. The density of $CD4^+$T cells in the portal area of BA2 group was significantly higher than BA1 and control groups ($p < 0.05$; **Figure 1C** and **Table 2**). When going to the subset level, both BA1 and BA2

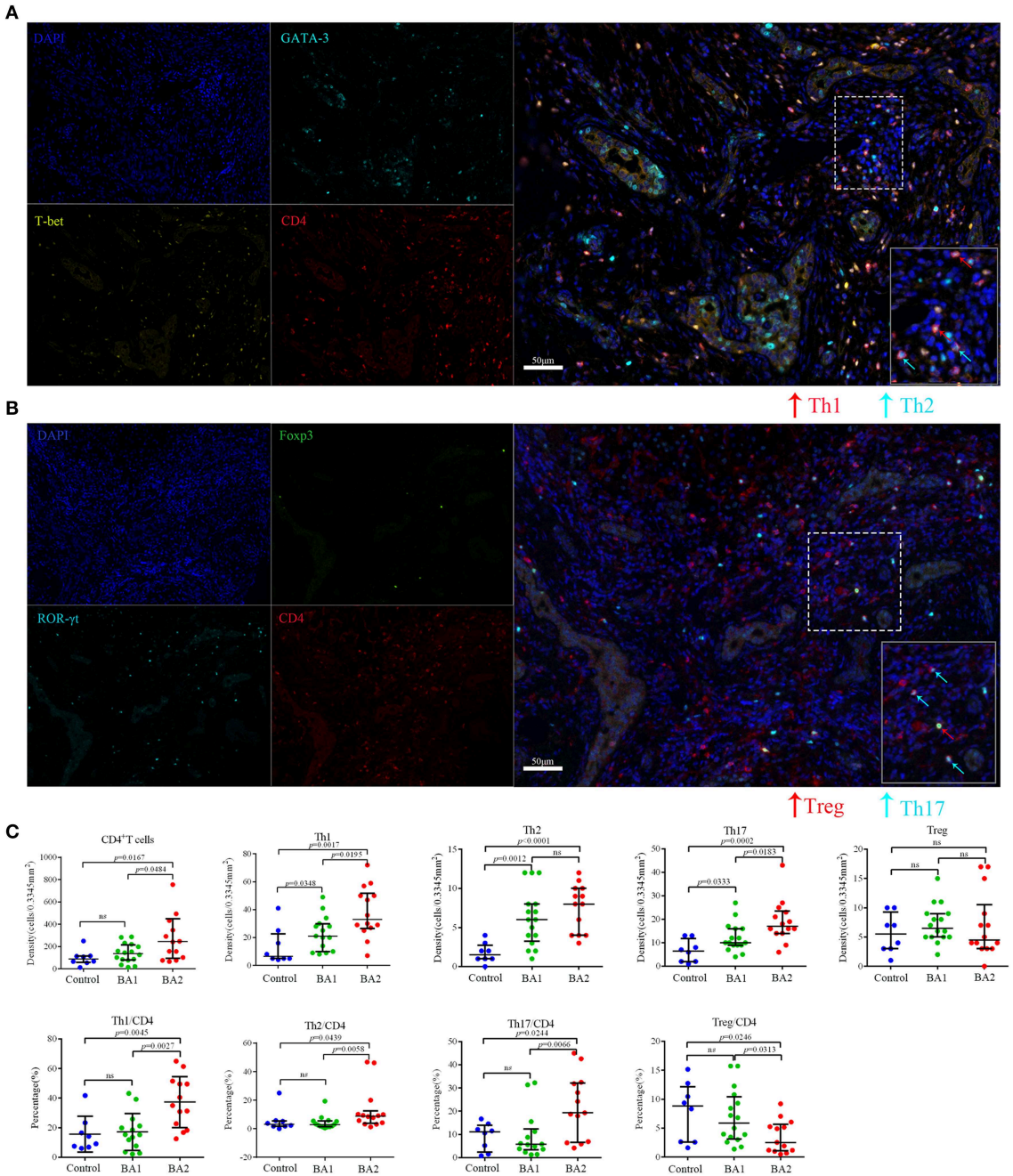

FIGURE 1 | Representative mIHC images and statistical analyses of $CD4^+$T cell subsets. **(A)** The four images on the left side were single stain of DAPI (blue), CD4 (red), T-bet (yellow), and GATA-3 (cyan). Merged image on the right side showed Th1 ($CD4^+$T-bet^+) and Th2 ($CD4^+$GATA3$^+$) cells. The lower right image in the merged image was the magnification of dotted line area. **(B)** The four images on the left side were single stain of DAPI (blue), CD4 (red), ROR-γt (cyan), and Foxp3 (green). Merged image on the right side showed Th17 ($CD4^+$ROR-γt^+) and Treg ($CD4^+$Foxp3^+) cells. The lower right image in the merged image was the magnification of dotted line area. **(C)** Analyses of the densities and percentages of $CD4^+$T cell subsets among control (choledochal cyst), BA1 (good prognosis) and BA2 (poor prognosis) groups. Mann-Whitney test was used.

TABLE 2 | The infiltrating density and percentage of major $CD4^+$T cell subsets.

	Control	BA1	BA2
$CD4^+$T cells density	87.0 (57.8–125.3)	135.0 (78.5–214.5)	244.0 (95.5–448.0)
Th1 density	6.5 (5.0–22.8)	23.0 (11.0–33.0)	33.0 (26.5–51.8)
Th1 percentage	11.6% (7.0–21.6%)	15.3% (7.1–24.7%)	37.7% (21.6–53.9%)
Th2 density	1.5 (1.0–2.8)	6.0 (3.3–8.0)	8.5 (4.0–10.3)
Th2 percentage	3.1% (1.8–5.6%)	2.8% (1.5–5.7%)	8.8% (4.0–12.6%)
Th17 density	6.5 (2.0–11.8)	10.0 (9.0–16.0)	17.0 (14.0–23.5)
Th17 percentage	11.1% (2.4–14.0%)	5.7% (3.7–14.5%)	21.8% (6.8–35.0%)
Treg density	5.5 (3.0–9.3)	6.5 (5.0–9.0)	4.5 (3.0–10.5)
Treg percentage	8.8% (2.6–12.2%)	5.9% (3.1–10.4%)	2.2% (0.9–5.5%)
$ICOS^+$Treg density	2.0 (0.5–3.75)	2.5 (0.3–4.5)	3.0 (0.8–8.3)
$ICOS^+$Treg percentage	29.3% (5.0–81.0%)	27.9% (5.0–55.6%)	65.3% (18.8–89.3%)
$ICOS^-$Treg density	2.0 (1.0–6.5)	4.0 (3.0–6.0)	2.0 (0–3.3)
$ICOS^-$Treg percentage	70.7% (19.1–95.0%)	72.1% (44.4–95.0%)	25.8% (25.8–72.3%)

Data were shown as the medians ± IQRs (inter-quartile range).
Density unit: cells/0.3345 mm^2; percentage: cells/CD4; Treg percentage: Treg subset/Treg. control, choledochal cyst; BA1, good prognosis; BA2, poor prognosis.

groups had higher densities of Th1, Th2, and Th17 cells than the control group. In addition, BA2 group displayed a higher density of Th1 and Th17 cells than BA1 group. We also compared the cell percentages and the results indicated that Th1, Th2, and Th17 percentages from BA2 group all significantly higher than BA1 and control groups ($p < 0.05$; **Figure 1C** and **Table 2**). Collectively, the portal area of BA liver was enriched with Th1, Th2, and Th17 cells and the infiltration levels were further increased in BA2 group, suggesting that an enhanced inflammation was linked to the deterioration of BA (density unit: cells/0.3345 mm^2; percentage: cells/CD4; Treg percentage: Treg subset/Treg).

Detection of Tregs and $ICOS^+$/$ICOS^-$Tregs in the Portal Area of Livers From BA Patients

With a similar study strategy as above, the infiltration of Tregs ($CD4^+Foxp3^+$) were detected in the livers of BA and control groups (**Figures S2E**, **S3D**). Though the densities of Tregs did not show significance among the three groups, the Treg percentage of BA2 group was significantly lower than the BA1 and control groups ($p < 0.05$; **Figure 1C**; **Table 2**).

According to the expression of ICOS, Tregs can be further divided into $ICOS^+$Tregs and $ICOS^-$Tregs, thus we proceeded to evaluate the expression of ICOS on Tregs by flow cytometry and found that the percentage of $ICOS^+$Tregs was increased in the peripheral blood of BA patients compared to the controls. Strikingly, $ICOS^+$Tregs were significantly increased and on average, was accounted for more than half of total Tregs in the livers of BA patients (**Figure 2A**). Next, we applied IHC to confirm the above detection, representative images were shown in **Figure S2F** and **Figure 2B**. The infiltrating densities and percentages of $ICOS^+$Tregs and $ICOS^-$Tregs in the portal areas were included in **Table 2**. Group comparison analysis revealed that the BA2 group showed a significant decrease of the density of $ICOS^-$Tregs compared to BA1 group ($p < 0.05$; **Figure 2C**). Furthermore, there was a tendency of increased percentage of $ICOS^+$Tregs and a concomitant decreased percentage of $ICOS^-$Tregs in the BA2 group (**Figure 2C**). These results suggested that the decreased number of hepatic Tregs particularly $ICOS^-$Tregs were already existed before operation which might correlate to the poor prognosis of the BA patients.

Correlation Between Infiltration of Hepatic Major CD4+T Cell Subsets and Clinical Indexes at Sixth Month After Surgery in BA Patients

To determine whether preoperative infiltrating $CD4^+$T cell subsets in the portal areas of BA livers had clinical relevance, correlation analysis was performed between infiltrating $CD4^+$T cell subsets and postoperative serum TB and DB levels at sixth month. Our data revealed that elevated total $CD4^+$T cells as well as percentages of Th1, Th2, and Th17 cells were positively correlated with these two serum parameters; on the contrary, Tregs percentage; and $ICOS^-$Tregs density showed negative correlation (All *p*-values were below 0.05, **Table 3**). These results suggested that the level of preoperative inflammatory response influenced the bile drainage after surgery and the time of jaundice-free survival. Whereas, hepatic Tregs as well as $ICOS^-$Tregs were favorable factors for bile drainage.

Prognostic Significance of Preoperative Infiltration of Major CD4+T Cell Subsets in BA Patients

Next, we evaluated the prognostic values of the $CD4^+$T cell subsets. The optimal cut-off for immunocytes infiltration was determined by ROC curve analysis (**Figure S4** and **Figure S5A**), and then each subset was divided into high and low groups according to the cut-offs. By using Kaplan–Meier curves, we identified that high $CD4^+$T cell, Th1, and Th17 densities, as well as high Th1, Th2, and Th17 percentages were negatively associated with jaundice-free and improved liver

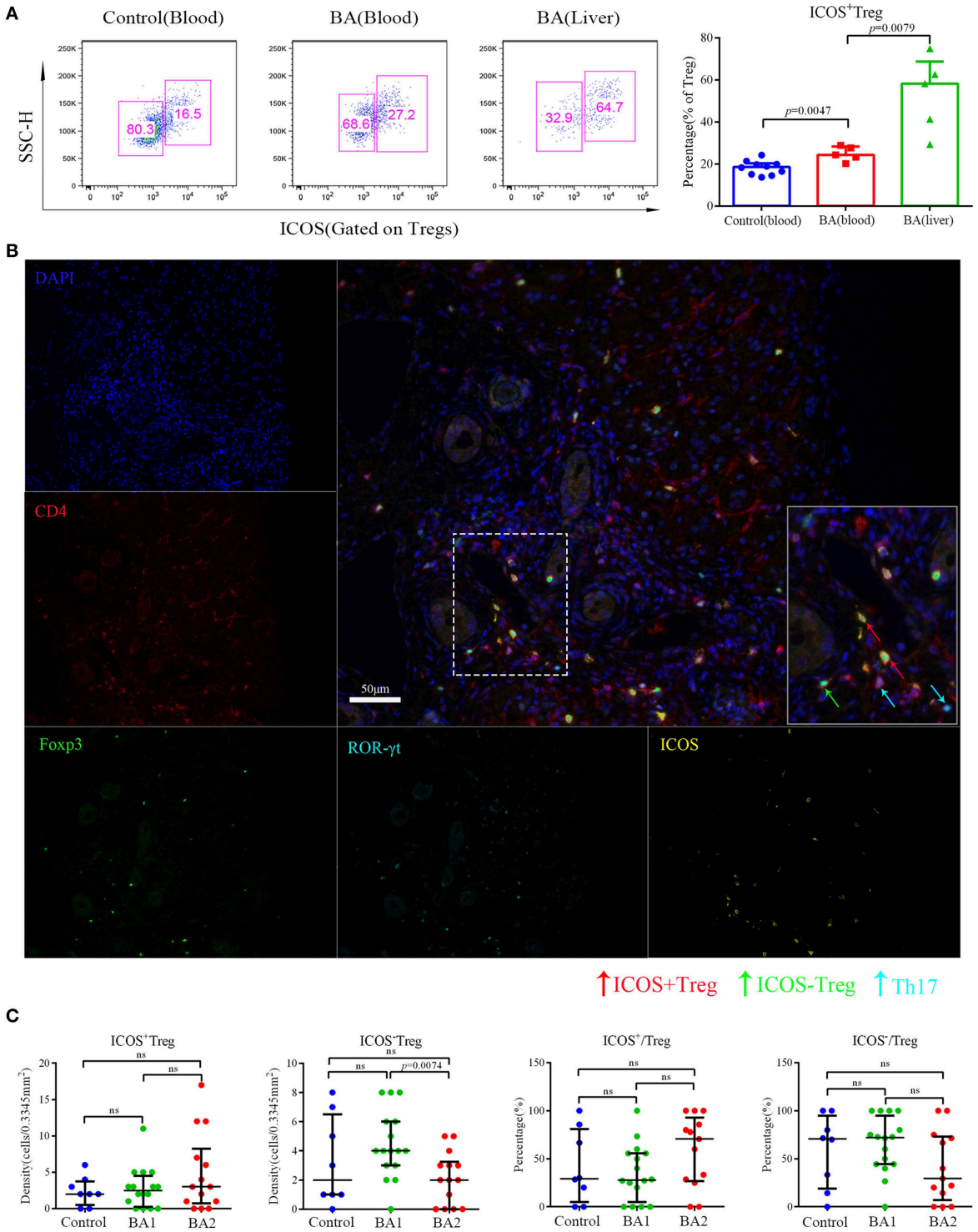

FIGURE 2 | Flow cytometry and mIHC analyses of Treg subsets. **(A)** Representative flow cytometry image (left) and statistical analysis (right) of ICOS expression on Tregs. Control patients included four patients with brachial plexus injury and six patients with accessory ear. **(B)** The five small images were single stain of DAPI (blue), CD4 (red), ROR-γt (cyan), Foxp3 (green), and ICOS (yellow). Merged image on the right upper side showed Th17 ($CD4^+$ ROR-$γt^+$) $ICOS^+$Treg ($CD4^+Foxp3^+ICOS^+$) and $ICOS^-$Treg ($CD4^+Foxp3^+ICOS^-$). The lower right image in the merged image was the magnification of dotted line area. **(C)** Analyses of the densities and percentages of Treg subsets among control (choledochal cyst), BA1 (good prognosis), and BA2 (poor prognosis) groups. Mann-Whitney test was used.

TABLE 3 | Correlation between infiltrating levels of immune cells and bilirubin levels at sixth month after surgery.

	TB	DB
$CD4^+$T cells density	**$r = 0.4069$ $p = 0.0256$**	**$r = 0.4220$ $p = 0.0202$**
Th1 density	$r = 0.1441$ $p = 0.4475$	$r = 0.1557$ $p = 0.4114$
Th1 percentage	**$r = 0.4082$ $p = 0.0279$**	**$r = 0.4271$ $p = 0.0208$**
Th2 density	$r = 0.3247$ $p = 0.0800$	**$r = 0.3707$ $p = 0.0437$**
Th2 percentage	**$r = 0.4680$ $p = 0.0105$**	**$r = 0.4998$ $p = 0.0058$**
Th17 density	$r = 0.3553$ $p = 0.0540$	**$r = 0.4017$ $p = 0.0278$**
Th17 percentage	**$r = 0.4501$ $p = 0.0143$**	**$r = 0.4801$ $p = 0.0084$**
Treg density	$r = -0.1377$ $p = 0.4682$	$r = -0.07796$ $p = 0.6822$
Treg percentage	**$r = -0.4225$ $p = 0.0200$**	**$r = -0.3749$ $p = 0.0412$**
$ICOS^+$Treg density	$r = 0.1202$ $p = 0.5270$	$r = 0.1620$ $p = 0.3924$
$ICOS^+$Treg percentage	$r = 0.2865$ $p = 0.1318$	$r = 0.3240$ $p = 0.0864$
$ICOS^-$Treg density	**$r = -0.4014$ $p = 0.0279$**	**$r = -0.3737$ $p = 0.0419$**
ICOS-Treg percentage	$r = -0.2865$ $p = 0.1318$	$r = -0.3240$ $p = 0.0864$

Density unit: cells/0.3345 mm^2; percentage: cells/CD4; Treg percentage: Treg subset/Treg.
Positive correlation: $r > 0$; negative correlation: $r < 0$.
DB, direct bilirubin; TB, total bilirubin. Bold values indicate the p-values < 0.05.

function survival (All p-values were below 0.05, **Figure 3A** and **Figure S5B**).

By contrast, a high Treg percentage was positively associated with BA prognosis ($p = 0.02$; **Figure 3B**). When Tregs were further divided into $ICOS^+$ and $ICOS^-$Tregs, only $ICOS^-$Treg percentage and density were positively associated with jaundice-free survival ($p = 0.013$ and 0.026, respectively; **Figure 3B** and **Figure S5B**). We further explored the prognostic value of the ratios of Treg or Treg subsets vs. Th17 cells. The results showed that only a high ratio of $ICOS^-$Treg to Th17 cells was positively associated with jaundice-free survival ($p = 0.005$; **Figure 3C**). Collectively, we demonstrated that a high infiltration of Th1, Th2, and Th17 cells were negatively associated with BA prognosis, while a high infiltration of Tregs was a favorable prognostic factor for BA which was ascribed to the $ICOS^-$Treg subset.

Univariate and Multivariate Analyses

To illustrate whether infiltrating $ICOS^+$Tregs and $ICOS^-$Tregs were independent prognostic factor, clinicopathologic features, and each $CD4^+$T cell subset showing significance by univariate analysis were adopted as covariates when performing multivariate Cox regression analysis (**Table 4**). However, univariate analysis concerning all the clinicopathologic features in BA did not appear significance. Thus, we adopted each $CD4^+$T cell subset showing significance in univariate analysis to consider those for multivariate evaluation. Patients with high infiltrating percentage of $ICOS^+$Tregs in the portal area harbored a 3.427-fold higher risk of persistent jaundice after operation compared with those patients with low percentage of $ICOS^+$Tregs (**Table 5**; HR, 3.427; 95%CI, 1.030–11.406; $p = 0.045$). For preoperative $ICOS^-$Tregs percentage, it was a protective factor in BA (**Table 5**; HR, 0.292; 95%CI, 0.088–0.971; $p = 0.045$).

Phenotypic and Molecular Analyses of $ICOS^+$ and $ICOS^-$Tregs From BA

The above results highlighted a possible role of $ICOS^-$Tregs in restricting BA progression. Through phenotypic analysis, we found more CD45RA and less CD45RO were expressed on peripheral blood and liver-derived $ICOS^-$Tregs than $ICOS^+$Tregs in BA patients ($p < 0.05$; **Figures 4A–C**), indicating that $ICOS^-$Tregs were in a less differentiated status. In addition, $ICOS^-$Tregs expressed more CD73 and less CD39 (**Figures 4A–C**), two molecules involved in adenosine metabolism and immune suppression (18). RT-qPCR results showed that $ICOS^-$Tregs expressed higher *CD25* and *TGF-β* but lower *CD39* than $ICOS^+$Tregs ($p < 0.05$; **Figure 4D**). $ICOS^-$Tregs also showed an increased expression of *CD73*, even though not reached significance (**Figure 4D**). Interestingly, we found that $ICOS^-$Tregs expressed higher anti-apoptotic molecule *BCL-2* (**Figure 4D**). And $ICOS^-$Tregs sorted from both the blood and livers of BA patients showed a much higher survival capacity than $ICOS^+$Tregs *in vitro* (**Figure 4E**). All these results indicated that $ICOS^-$Tregs were different from $ICOS^+$Tregs in several aspects, including less differentiation, high expression of certain suppressive molecules and increased capacity to survive *in vitro*.

Increased Capacity of $ICOS^-$Tregs to Suppress Cytokines Production of Effector T Cells

To compare the suppressive activities of $ICOS^+$ and $ICOS^-$Tregs, we sorted both the subsets and cocultured each of them with $CD3^+CD25^-$T cells. Because insufficient Tregs count in a small hepatic tissue could not meet the needs of the experiment, we had to choose the Tregs from peripheral blood to mimic the closest setup for a replacement. Representative images were shown in **Figures 5A,B**. The results indicated that compared to $ICOS^+$Tregs, $ICOS^-$Tregs exhibited a stronger capacity to inhibit the production of TNF-α, IL-2, and IFN-γ from $CD4^+$T cells (**Figure 5C**), as well as TNF-α and IL-2 production from $CD8^+$T cells (**Figure 5D**). While the production of IFN-γ from $CD4^+$T cells increased in the $ICOS^+$Treg co-culture system instead of decrease (**Figure 5C**). These results demonstrated that $ICOS^-$Tregs were more suppressive under this experimental condition which provided a possible explanation for their beneficial effect on prognosis.

DISCUSSION

In the present study, we accurately quantitated the infiltrating $CD4^+$T cell subsets in the portal areas of BA livers and found that preoperative high infiltrating Th1, Th2, and Th17 cells were harmful to BA patients. In contrast, Tregs had a protective role in this disease, especially $ICOS^-$Tregs, as they could effectively suppress the production of harmful cytokines in BA and were further proved to be a favorable prognostic factor.

Previous studies had proved the pathogenic roles of Th1, Th2, and Th17 cells in BA separately by confirming that IFN-γ (19, 20), IL-13 (21), and IL17 (22) were the main pathogenic

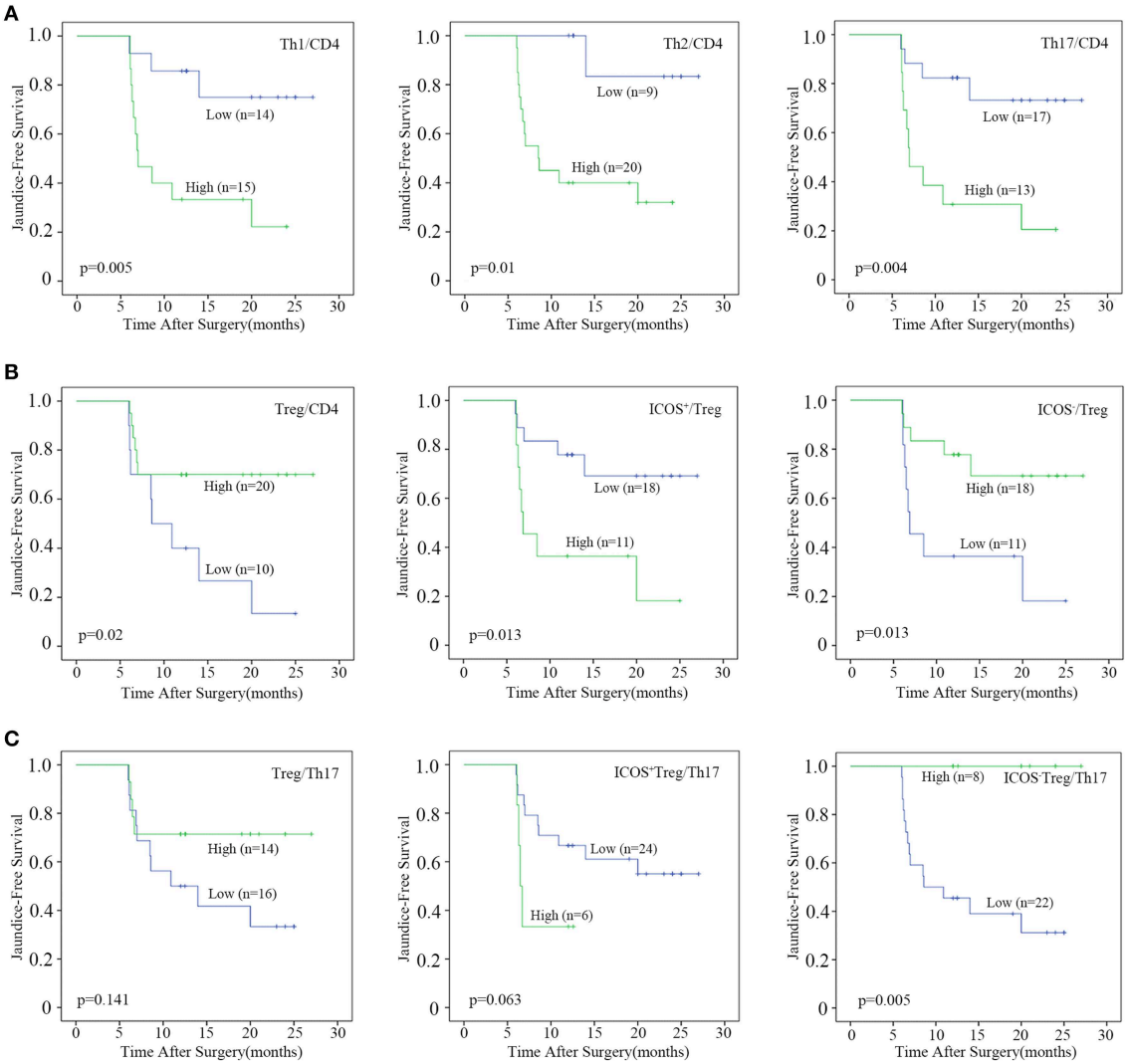

FIGURE 3 | Prognostic analyses of CD4$^+$T cell subsets. Patients were divided into "high" and "low" groups according to the optimal cut-offs which were determined by ROC curve analysis. Kaplan–Meier analysis of jaundice-free survival for **(A)** the percentages of Th1, Th2, and Th17 cells, **(B)** the percentages of Treg, ICOS$^+$Treg, and ICOS$^-$Treg cells, **(C)** the ratio of Treg, ICOS$^+$Treg, and ICOS$^-$Treg to Th17 were analyzed. Log-rank test was used.

cytokines. Most recently, a hypothesis raised by Bezerra et al. (23) emphasized that there might exist biological transitions from a type 1 to a mixed (Th1-2-17) immune response that drives persistent liver injury and progressive fibrosis in BA. This hypothesis is proved in this study. First, we revealed that both Th1 and Th17 cells were significantly enriched in BA livers, which increased even higher in severe cases. Secondly, we found Th2 cells also increased in BA livers, despite previous research showing that Th1 and Th2 cells remain a dynamic balance *in vivo* normally, overactivation of either subset can cause disease, and either pathway can down-regulate the other (24). Notably, the enhanced Th2 response was not only detected in a minority of BA patients or was an inflammatory stage that independent of Th1 response but existed in conjunction with the Th1 response. Thus, our clinical research revealed that a globally elevated preoperative effector CD4$^+$T cell response could result in totally different prognosis in BA patients with similar preoperative clinical features.

The CD28 family member ICOS is important in regulating the development and immunosuppressive function of Tregs and is an immunological hotspot in tumor immunology (25, 26). Here, we exclusively proved the existent of both ICOS$^+$Tregs and ICOS$^-$Tregs in BA. Previously reported, ICOS$^+$Tregs are more suppressive than ICOS$^-$Tregs (27). However, ICOS$^-$Tregs, instead of ICOS$^+$Tregs exhibited a more powerful capacity to suppress the overproduction of harmful cytokines [e.g., IFN-γ, TNF-α, and IL-2 (28)] and were more beneficial to prognosis in our study. We conclude the possible reasons as followed: First, ICOS$^-$Tregs expressed more CD73 and less CD39 compared with ICOS$^+$Tregs. CD39 is an ectoenzyme

TABLE 4 | Univariate analyses of prognostic factors associated with jaundice-free survival ($n = 30$).

Variables (cutoffs*)	HR	95%CI	*p*-value
Gender (M vs. F)	0.866	0.290–2.586	0.797
Operative age, days (>60 vs. ≤60)	0.914	0.203–4.104	0.906
Weight, kg (>4 vs. ≤4 and >2.5)	0.589	0.183–1.889	0.373
PLT, U/L (>300 vs. ≤300)	1.267	0.438–3.669	0.662
AST, U/L (>50 vs. ≤50)	23.037	0.006–93618.311	0.457
ALT, U/L (>50 vs. ≤50)	2.535	0.331–19.396	0.370
DB, μmol/L (>96.4 vs. ≤96.4)	0.481	0.166–1.390	0.176
TB, μmol/L (>140 vs. ≤140)	0.382	0.132–1.105	0.076
GGT, U/L (>300 vs. ≤300)	0.815	0.282–2.354	0.705
$CD4^+$T cell density (high vs. low)	4.188	1.442–12.167	**0.008**
Th1 cell density (high vs. low)	3.140	0.983–10.032	0.053
Th1 cell percentage (high vs. low)	5.175	1.427–18.758	**0.012**
Th2 cell density (high vs. low)	2.476	0.819–7.483	0.108
Th2 cell percentage (high vs. low)	9.134	1.189–70.198	**0.034**
Th17 cell density (high vs. low)	5.986	1.337–26.804	**0.019**
Th17 cell percentage (high vs. low)	4.752	1.476–15.302	**0.009**
Treg cell density (high vs. low)	2.212	0.775–6.311	0.138
Treg cell percentage (high vs. low)	0.304	0.105–0.879	**0.028**
$ICOS^+$Treg cell density (high vs. low)	4.830	1.471–15.860	**0.009**
$ICOS^+$Treg cell percentage (high vs. low)	3.815	1.231–11.825	**0.020**
$ICOS^-$Treg cell density (high vs. low)	0.257	0.071–0.930	**0.038**
$ICOS^-$Treg cell percentage (high vs. low)	0.262	0.085–0.812	**0.020**

Density unit: cells/0.3345 mm^2; percentage: cells/CD4; Treg percentage: Treg subset/Treg.

***Cutoffs*:** operative age, weight, PLT, AST, and ALT were set based on normal or abnormal; DB, TB, GGT, cell percentages, and cell densities were set based on Youden index.*

M, male; F, female; PLT, Platelets; AST, aspartate aminotransferase; ALT, alanine aminotransferase; DB, direct bilirubin; TB, total bilirubin; GGT, gamma-glutamyl transpeptidase; HR, hazard ratio; CI, confidence interval. Bold values indicate the p-values < 0.05.

that could hydrolyze ATP and ADP to AMP, while CD73 is an ecto-5′-nucleotidase that converts AMP to adenosine which could suppress effector $CD4^+$T cell response and cytokines secretion (18, 29). Consistently, Sauer et al. (30) found that Tregs with decreased CD39 and increased CD73 expression in $ADA^{+/+}$ mice were more suppressive than Tregs expressed the opposite pattern in $ADA^{-/-}$ mice. Second, $ICOS^-$Tregs which expressed more CD45RA but less CD45RO were more resistant to apoptosis due to their less differentiated status (31). Besides, this subset also upregulated BCL-2 expression which could inhibit cell apoptosis (32). Therefore, $ICOS^+$Tregs might not be able to persistently sustain immune balance as $ICOS^-$Tregs in BA due to higher apoptosis. Third, we confirmed higher expression of three immunosuppression-related genes CD25, CD73, and TGF-β in $ICOS^-$Tregs which might enable $ICOS^-$Tregs to perform a more suppressive function in BA. Similar phenomena were also demonstrated in a previous study that $KLRG1^+ICOS^+$Tregs were prone to apoptosis, and had an impaired proliferative capacity and suppressive function (33), but $KLRG1^+$ and $KLRG1^-$Treg subsets generally displayed a similar suppressive potential (34). $KLRG1^+ICOS^+$Tregs could even reprogram into inflammatory cytokine-producing effector T cells (33), and $ICOS^+$Tregs adopted a Th1-like Treg phenotype could produce more IFN-γ (35). These might also explain the enhanced IFN-γ production in $ICOS^+$Treg co-culture system.

Clinically, BA patients usually receive sequential therapy of KPE and liver transplantation. However, whether all the patients should firstly receive KPE is under controversy because a successful KPE could not restore the impaired liver function of BA patients who already have severe cirrhosis and liver inflammation before operation. Therefore, it is necessary to predict the prognosis of BA patients and perform a KPE on those selective patients who may get benefit from it. Serum GGT levels (36) and transient elastography (37) were applied in predicting

TABLE 5 | Multivariate analyses of prognostic factors associated with jaundice-free survival ($n = 30$).

Variables*	Univariate	A†		B†		C†	
	p	HR (95%CI)	*p*	HR (95%CI)	*p*	HR (95%CI)	*p*
Densities of $CD4^+$T cell subsets							
$CD4^+$T cell density (high vs. low)	**0.008**	2.335 (0.758–7.195)	0.140	2.624 (0.886–7.771)	0.082		
Th17 cell density (high vs. low)	**0.019**	4.015 (0.853–18.898)	0.079	3.555 (0.751–16.828)	0.110		
$ICOS^+$Treg cell density (high vs. low)	**0.009**	2.952 (0.873–9.983)	0.082				
$ICOS^-$Treg cell density (high vs. low)	**0.038**			0.460 (0.123–1.716)	0.247		
Percentages of $CD4^+$T cell subsets							
Th1 cell percentage (high vs. low)	**0.012**	2.478 (0.200–30.649)	0.480	0.626 (0.051–7.708)	0.715	0.626 (0.051–7.708)	0.715
Th2 cell percentage (high vs. low)	**0.034**	4.100 (0.366–45.914)	0.252	4.394 (0.394–48.963)	0.229	4.394 (0.394–48.963)	0.229
Th17 cell percentage (high vs. low)	**0.009**	0.973 (0.109–8.667)	0.980	2.966 (0.354–24.859)	0.316	2.966 (0.354–24.859)	0.316
Treg cell percentage (high vs. low)	**0.028**	0.361 (0.117–1.117)	0.077				
$ICOS^+$Treg cell percentage (high vs. low)	**0.020**			3.427 (1.030–11.406)	**0.045**		
$ICOS^-$Treg cell percentage (high vs. low)	**0.020**					0.292 (0.088–0.971)	**0.045**

***Variables*:** variables which showed significance in **Table 4** (univariate analyses) were selected and divided into densities group and percentages group.*

Column† A, B, and C were multivariate analyses performed by Cox regression analysis. Tregs or Treg subsets were analyzed with the other covariates which showed significance to determine whether they were independent prognostic factors.

HR, hazard ratio; CI, confidence interval. Bold values indicate the p-values < 0.05.

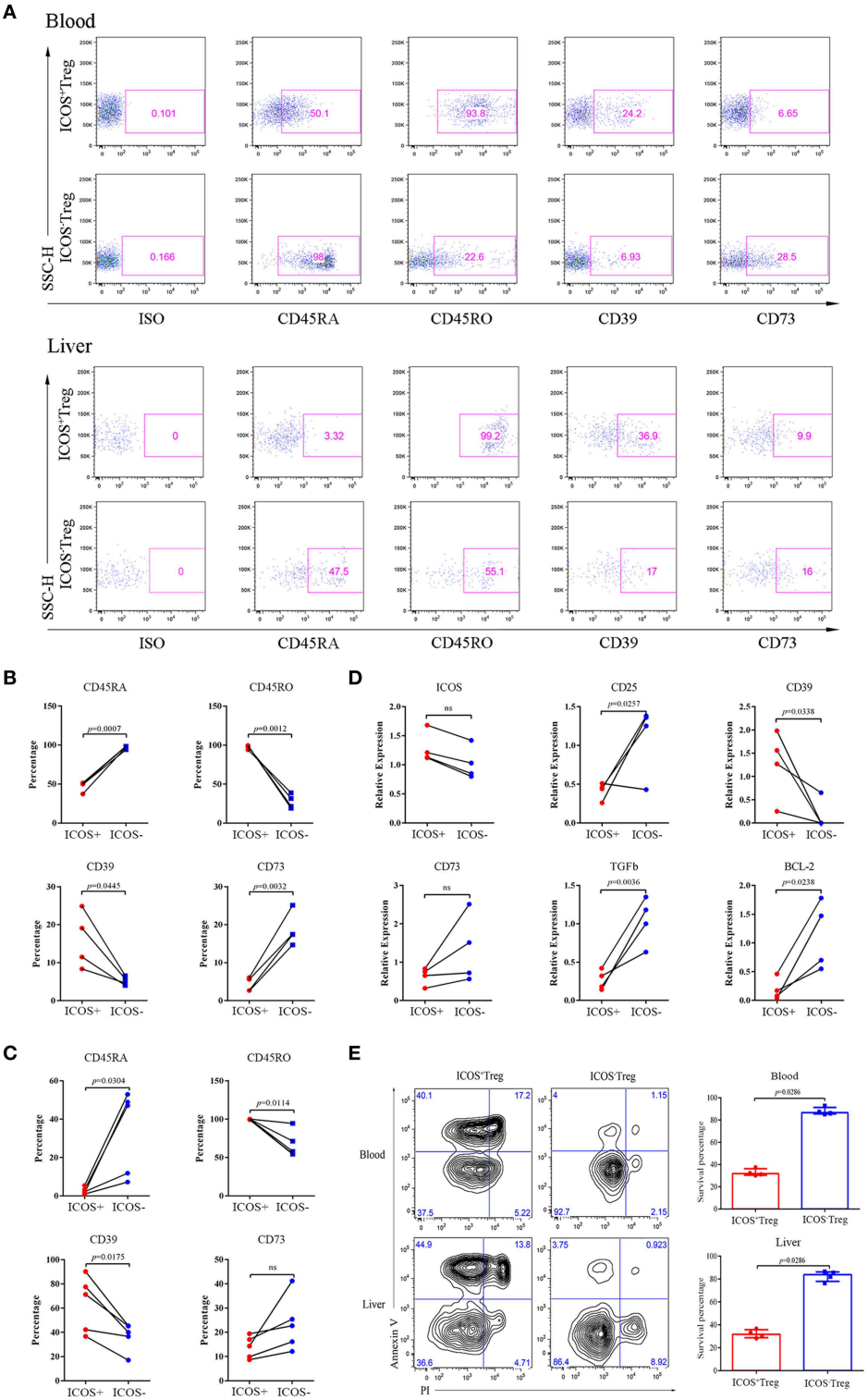

FIGURE 4 | Phenotypes, RT-qPCR and apoptosis analyses of ICOS^{+}Tregs and ICOS^{-}Tregs. **(A)** Representative images of phenotypic expression of Treg subsets in peripheral blood (upper) and liver tissue (lower) from BA patients. Gates were set based on the ISO. **(B,C)** The statistical analysis of phenotypic expression of Treg subsets from peripheral blood (upper) and liver tissue (lower). Wilcoxon test was used. **(D)** RT-qPCR validation of *ICOS*, *CD25*, *CD39*, *CD73*, *TGF-β*, and *BCL-2* genes were analyzed. Wilcoxon test was used. **(E)** Apoptosis Assay of Treg subsets from peripheral blood (upper) and liver tissues (lower) of BA patients. Mann-Whitney test was used.

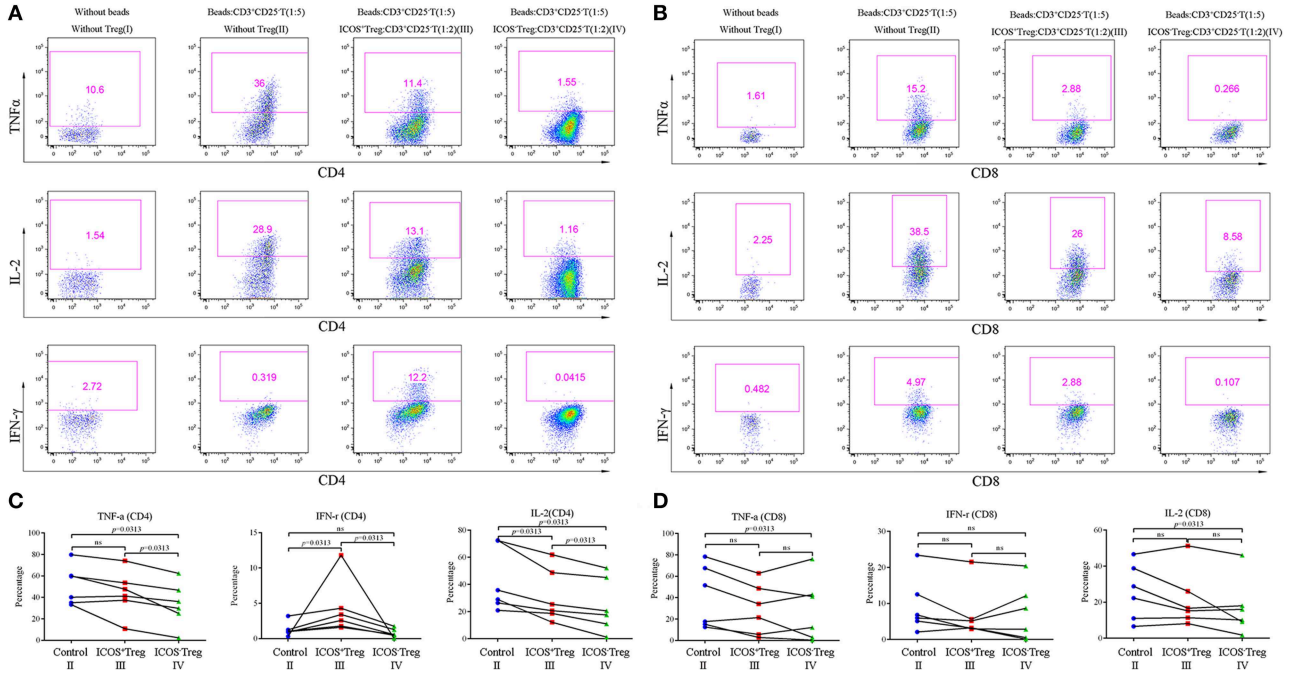

FIGURE 5 | Co-culture experiments of $CD3^+CD25^-$T cells and Treg suubsets. **(A,B)** Effector $CD3^+CD25^-$T cells were cultured with or without Treg subsets and $CD3^+CD28^+$ dynabeads (ratios were shown in the parenthesis) for 3 days, the representative flow cytometry images of cytokines secretion were shown as above. **(C,D)** Cytokines including TNF-α, IL-2, and IFN-γ produced by $CD4^+$ and $CD8^+$T cells in the control (II), $ICOS^+$Treg (III), and $ICOS^-$Treg (IV) systems were analyzed. Wilcoxon test was used.

the prognosis of BA, however their accuracy varied. Postoperative serum TB and DB levels were associated with BA prognosis, especially TB, 2014 Practice Guideline of American Association for the Study of Liver Diseases (AASLD) indicated that BA patients should be promptly referred for liver transplantation evaluation if the TB was >6 mg/dL beyond 3 months after KPE (38). We herein found that the preoperative infiltrating effector $CD4^+$T cells were positively correlated the serum TB and DB levels at the sixth month after surgery and were negatively associated with patients' prognosis. Thus, we supported the early detection of $CD4^+$T lymphocytes as an important reference for postoperative liver function and prognosis. It should be pointed out that $ICOS^-$Treg percentage which was an independent prognostic factor, especially the ratio of $ICOS^-$Treg to Th17 cells was a better predictive measure for prognosis. Together, we provided a potential new biomarker to predict the prognosis of BA patients.

We believe that the strong inference of this study needs to be encouraged to perform in larger cohorts combining immunopathological aspect, and will ensure therapeutic interpretability and stratification accuracy. In summary, we here prove that BA is an immune-related disease and preoperative immune dysfunction is one of the triggers which could aggravate the condition of BA patients. In future, adjuvant immunotherapy may have the potential to alleviate the symptom or delay the progression of BA if we find the right target.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

Statement involving human subjects: This study was carried out in accordance with the recommendations of the Ethics Committee of the Children's Hospital of Fudan University (2018 [164]) with written informed consent in accordance with Declaration of Helsinki. The protocol was approved by the Ethics Committee of the Children's Hospital of Fudan University.

AUTHOR CONTRIBUTIONS

GC and XZ designed the study and interpreted the data. ShuZ did the experiments, analyzed the data, and wrote the manuscript. SG analyzed and interpreted the data. JM did the experiments and analyzed the data. LM interpreted the data and wrote the manuscript. YW gave the technical support. FZ analyzed the data. DZ did the experiments. ShaZ, RD, and XX interpreted the data. All authors gave approval for the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2019.00279/full#supplementary-material

Figure S1 | Sorting gates of ICOS$^+$Tregs, ICOS$^-$Tregs, and CD3$^+$CD25$^-$T cells in BD FACSMelody flow cytometric cell sorter. **(A)** Sorting gates of ICOS$^+$Tregs and ICOS$^-$Tregs. **(B)** Sorting gate of CD3$^+$CD25$^-$T cells.

Figure S2 | Representative images of single immunohistochemistry. Antibodies tests of single immunohistochemistry of **(A)** CD4, **(B)** T-bet, **(C)** GATA-3, **(D)** ROR-rt, **(E)** Foxp3, **(F)** ICOS in the portal areas of BA livers.

Figure S3 | Representative images of multiplex immunohistochemistry. The lower right image is the magnification of dotted line area. Triple immunohistochemistry of **(A)** DAPI (blue), CD4 (red), and T-bet (cyan) to identify Th1 (CD4$^+$T-bet$^+$) cells, **(B)** DAPI (blue), CD4 (red), and GATA-3 (yellow) to identify Th2 (CD4$^+$GATA-3$^+$) cells, **(C)** DAPI (blue), CD4 (red), and ROR-γt (cyan) to identify Th17 (CD4$^+$ROR-γt$^+$) cells, **(D)** DAPI (blue), CD4 (red), and Foxp3 (green) to identify Treg (CD4$^+$Foxp3$^+$) cells.

Figure S4 | ROC curve analyses of CD4$^+$T cell subsets. **(A)** Univariate ROC curve analysis about prognostic outcome onto Th1, Th2, and 687 Th17 percentages. **(B)** Univariate ROC curve analysis about prognostic outcome onto 688 Treg and Treg subsets percentages. **(C)** Univariate ROC curve analysis about 689 prognostic outcome onto ratios of Treg or Treg subsets to Th17.

Figure S5 | ROC curve and Kaplan-Meier analyses for the density of CD4$^+$T cell subsets. **(A)** Univariate ROC curve analysis about prognostic outcome onto density of each CD4$^+$T cell subset was performed. **(B)** Kaplan–Meier curves comparing jaundice-free survival and improved liver function in patients with high and low densities of CD4, Th1, Th2, Th17, Treg, ICOS$^+$Treg, and ICOS$^-$Treg cells in the portal areas were analyzed. Log-rank test was used.

Table S1 | Antibody list of immunohistochemistry.

Table S2 | Antibody list of flow cytometry.

Table S3 | Sequences of primers used for qPCR assays in this study.

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ORIGINAL RESEARCH
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Prevalence of Anemia and Its Associated Risk Factors Among 6-Months-Old Infants in Beijing

*Qinrui Li, Furong Liang, Weilan Liang, Wanjun Shi and Ying Han**

Department of Pediatrics, Peking University First Hospital, Beijing, China

Objective: The worldwide prevalence of anemia is ∼24.8%. Iron deficiency anemia is common in children and women and associated with sensory, motor, cognitive, language, and socioemotional deficits. Therefore, detection and early intervention strategies for anemia in infants are urgently needed. To prevent the occurrence of iron deficiency anemia, we aimed to identify risk factors associated with anemia in infants.

Methods: This investigation involved a cross-sectional study of 6-months-old infants discharged between April 2014 and September 2017 from Peking University First Hospital. We assessed birth information, maternal age, and maternal educational level as well as data on feeding style, complementary foods and primary caregivers. The infants were assessed with the Denver Developmental Screening Test (DDST).

Results: A total of 1,127 6-months-old infants were enrolled at the hospital. We found that the prevalence of anemia among infants in Beijing was ∼11.8%. Premature infants had a higher rate of anemia than full-term infants ($\chi^2 = 40.103$, $P < 0.001$). Infants born in autumn or winter were at an elevated risk of developing anemia ($\chi^2 = 22.949$, $P < 0.001$). Birth weight had no effect on the rate of anemia in infants ($\chi^2 = 0.023$, $P = 0.568$). Infants who were exclusively breastfeeding had higher anemia rates than those who were fed formula ($\chi^2 = 38.466$, $P < 0.001$). Infants whose caregivers added no complementary foods had higher anemia rates (24.7%) than those whose caregivers added more than two kinds of complementary food (8.2%). The type of caregiver had no effect on the anemia rate in infants ($\chi^2 = 0.031$, $P = 1.000$).

Conclusions: The following factors resulted in a higher prevalence of anemia in our study a gestational age at birth of <37 weeks, exclusive breastfeeding, a lack of supplementation with complementary foods and a spring birth date. No significant differences in DDST pass rates were evident between infants with and without anemia.

Keywords: iron deficiency anemia, growth and development, infants, Denver Development Screen Test (DDST), feeding style

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Edited by:
Ron Shaoul,
Rambam Health Care Campus, Israel

Reviewed by:
Corentin Babakissa,
Université de Sherbrooke, Canada
Tudor Lucian Pop,
Iuliu Haţieganu University of Medicine and Pharmacy, Romania

****Correspondence:***
Ying Han
hanying1568@126.com

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BACKGROUND

Anemia is a common disease that affects ∼1.6 billion people worldwide, especially infants and women. The World Health Organization (WHO) has estimated that the global prevalence of anemia to be ∼24.8% (1). Anemia is defined as a hemoglobin (Hb) concentration that is two standard deviations below the mean for the patient's age. The factors associated with anemia may include genetics, chronic infections, and nutritional deficiencies, such as hemoglobinopathies, iron deficiency, folate deficiency, and vitamin B12 deficiency. Iron deficiency anemia is common in children, and iron deficiency has a very important influence on infant neurological development. Iron is an essential factor in neuronal myelination, metabolism, neurotransmission and neurogenesis, and it affects behavior, memory, learning and sensory systems (2, 3). In rodents, iron deficiency alters the metabolome in the striatum and delays behavioral development (4). Iron deficiency also alters the neurochemical profile associated with cognitive function in the developing hippocampus (5). Iron deficiency in infancy is associated with impaired mental and motor development, especially in language capabilities, bodily balance and ordination skills (6, 7). Morath and Mayer-Proschel (8) found that iron deficiency during pregnancy affected the function of glial precursor cells in rats. Iron is essential for multiple enzymes associated with the synthesis of neurotransmitters, including dopamine and norepinephrine, which are associated with learning and memory function (9). Iron is important for multiple electron transfer reactions associated with brain energy metabolism (10). Perinatal iron deficiency reduces neuronal activity, especially in the hippocampal region, which is associated with memory function (11).

Infants aged 6–12 months are at an elevated risk of anemia because they are developing and growing rapidly and because the stored iron from the mother may be deficient. The addition of complementary food during this period is important. Complementary foods influence the overall nutritional status of the infant. The risk of iron deficiency increases in later infancy if infants are exclusively breastfeeding (12). A study of infants in poor rural areas of China showed that complementary food supplements could reduce the prevalence of anemia (13). Moreover, home food fortification with iron increased Hb levels and decreased anemia rates (14). Hong et al. (15) showed that the combination of prolonged breastfeeding and an inadequate supply of red meat results in iron deficiency and iron deficiency anemia. Insufficient complementary feeding behavior is associated with undernutrition, which results in poor growth and cognitive development. Baye et al. (16) found that positive, responsive maternal feeding behavior was positively associated with Hb concentrations. Other factors also influence the iron status of infants; one example is the maternal iron status, which is associated with anemia in children. In this case, anemia occurs because an infant cannot obtain enough iron from the stored iron transferred from the mother or from breast milk (17). The educational level of the mother or caregiver is also associated with the anemia rate (18, 19).

Rapid economic development and the acceleration of industrialization in China have led to major changes in Chinese lifestyles, especially for new parents. As the capital city of China, Beijing is more representative of such changes than other cities. Infants in Beijing consume increasingly rich diets, and their developmental health is better now than in the past. As the education level of the mother increases, her knowledge of how to feed her children improves. In the present study, we aimed to investigate the factors currently associated with infant anemia in Beijing, with the goal of improving the health of these infants.

METHODS

Participants

This investigation involved a cross-sectional study conducted at Peking University First Hospital. The participants were enrolled between April 2014 and September 2017. The infants included in this study were 6 months old and did not have severe disease or any abnormality at birth. The exclusion criteria were as follows: younger or older than 6 months old, a history of asphyxia at birth, and a history of severe disease. Children who met the inclusion criteria and did not meet the exclusion criteria were enrolled in our study. For each infant, we collected data regarding sex (male or female), maternal education level (less than undergraduate, undergraduate, or more than undergraduate), birth weight, birth season, caregivers (parent, grandparent, or babysitter), feeding style (exclusive breastfeeding, mixed feeding or formula feeding), and complementary food usage (none, one kind of complementary food or two or more kinds of complementary food). We defined the four seasons as follows: winter (December, January, and February), spring (March, April, and May), summer (June, July, and August), and autumn (September, October, and November).

Diagnostic Criteria and Classification

Anemia is defined as an Hb concentration <110 g/L according to the WHO diagnostic criteria. Mild anemia is defined as an Hb concentration between 90 and 110 g/L, moderate anemia is defined as an Hb concentration between 60 and 90 g/L, and severe anemia is defined as an Hb concentration <60 g/L. Iron deficiency anemia is defined as a mean cell volume (MCV) <80 fl, mean cell hemoglobin (MCH) <27 pg, and mean corpuscular Hb concentration (MCHC) <310 g/L.

Assessment of Ability Development

The development of the infants' intelligence was assessed with the Denver Developmental Screening Test (DDST). The DDST was standardized for Chinese use in 1982 and has been utilized worldwide to assess the intelligence development of children aged 1 month to 6 years. The standardized DDST consists of 104 items and covers four areas of development: (a) personal/social, (b) fine motor/adaptive, (c) language, and (d) gross motor. In the present study, three trained professionals examined the children. The response options for the items were "passes," "fails," "refuses," and "has not had the opportunity." The results of the DDST could be normal (no delays), suspect (2 or more caution items and/or 1 or more delays), abnormal (2 or more delays) or untestable (refusal

TABLE 1 | Infant birth information.

Sex	*N*	Birth weight (kg)	Birth length (cm)	Hemoglobin (g/L)
Male	591	3.38 ± 0.42	50.6 ± 2.1	117.9 ± 8.5
Female	536	3.26 ± 0.45	50.2 ± 1.8	118.4 ± 7.8

of one or more items completely to the left of the age line or more than one item intersected by the age line in the 75–90% area). The children with suspect or abnormal results were retested 2 or 3 weeks later.

Statistical Analysis

The data were analyzed with SPSS 18.0. Numerical variables are presented as the mean ± standard deviation (SD) (birth weight). Enumeration data and ranked data are presented as percentages. ANOVA, the χ^2 test and non-parametric tests were used to assess the differences in child development between the three groups. A *P*-value <0.05 was considered statistically significant.

Ethics

The study was carried out in accordance with recommendations of the Clinical Research Ethics Committee of Peking University First Hospital (Permit Number: 2017 [1375]). All parents provided written informed consent before the start of the study.

RESULTS

A total of 1,127 infants (591 male and 536 female) aged 6 months were included in this study. The average birth weights of the infants were 3.38 ± 0.42 kg for males and 3.26 ± 0.45 kg for females. The average birth lengths were 50.6 ± 2.1 cm for males and 50.2 ± 1.8 cm for females. The average Hb levels were 117.9 ± 8.5 g/L in males and 118.4 ± 7.8 g/L in females (**Table 1**). The mean maternal age was ∼31.8 ± 3.5 years.

Table 2 contains the demographic information (e.g., maternal educational level, maternal age, gestational age at birth, and birth season) and Hb levels of the included infants. The mean Hb value was 118.2 ± 8.1 g/L (range 80.0–146.0 g/L). A total of 133 (11.8%) infants had microcytic hypochromic anemia (MCV <80 fl, MCH <27 pg, and MCHC <310 g/L), including 126 (11.2%) with mild anemia (104.6 ± 4.7 g/L) and 7 (0.6%) with moderate anemia (85.3 ± 3.1 g/L). No infants displayed severe anemia. The mean Hb level in the non-anemia group was 120.1 ± 6.1 g/L. The mean Hb values of the groups with maternal educational levels of less than undergraduate, undergraduate and more than undergraduate were 118.0 ± 8.9, 118.7 ± 7.5, and 117.4 ± 8.5 g/L, respectively. The ages of the mothers ranged from 22 to 45 years old. The effects of maternal age on the Hb levels of the infants are shown in **Table 2**. The study group contained 65 premature infants, whose mean Hb level was 113.3 ± 10.3 g/L. The study group contained 1,062 full-term infants, whose mean Hb level was 118.5 ± 7.9 g/L. **Table 2** also shows the effects of birth season and birth weight on Hb levels.

As shown in **Table 3**, feeding practices affected the infants' Hb levels. A total of 197 (17.5%) infants were fed formula and had a mean Hb level of 120.7 ± 7.3 g/L. A total of 634 (56.3%) infants were exclusively fed breast milk and had a mean Hb level of 116.6 ± 8.5 g/L. A total of 296 (26.3%) infants received mixed feeding and had a mean Hb level of 119.9 ± 7.0 g/L. Most infants (96.3%) had diets containing complementary foods as follows: one type of complementary food (rice flour) or two or more types of complementary foods (rice flour, yolk or liver paste). A total of 202 (41.6%) infants, 253 (52.1%) infants, and 31 (6.4%) infants were cared for by their parents, grandparents and babysitters, respectively, and the mean Hb levels of these infants were 117.3 ± 7.6, 117.7 ± 8.2, and 118.3 ± 9.7 g/L, respectively.

TABLE 2 | Demographic information and hemoglobin levels.

Characteristics	*n*	Percent (%)	Hemoglobin (g/L)
Hemoglobin (g/L)			
Normal (>110)	994	88.2%	120.1 ± 6.1
Mild (90–109)	126	11.2%	104.6 ± 4.7
Moderate (60–89)	7	0.6%	85.3 ± 3.1
Severe (<59)	0	0	–
Maternal educational level			
Less than undergraduate	185	17.6%	118.0 ± 8.9
Undergraduate	580	55.2%	118.7 ± 7.5
More than undergraduate	285	27.1%	117.4 ± 8.5
Maternal age			
<25 years	8	0.7%	114.9 ± 6.6
25–29 years	289	26.9%	118.3 ± 8.1
30–34 years	556	51.8%	118.6 ± 7.8
35–39 years	187	17.4%	117 5 ± 8.4
>39 years	33	3.1%	116.8 ± 8.7
Gestational age at birth			
<37 weeks	65	5.8%	113.3 ± 10.3
>37 weeks	1,062	94.2%	118.5 ± 7.9
Birth season			
Spring	220	19.5%	117.1 ± 7.4
Summer	292	25.9%	118.1 ± 5.3
Autumn	336	29.8%	118.3 ± 9.5
Winter	279	24.8%	119.0 ± 9.2
Birth weight			
<2,500 g	36	3.3%	118.5 ± 9.5
>2,500 g	1,063	96.7%	118.1 ± 8.1

The factors that affected infant anemia are shown in **Table 4**. Gestational age at birth, birth season, feeding style and complementary food supplementation had clear effects on infant anemia. Premature infants had higher rates of anemia than full-term infants ($\chi^2 = 40.103$, $P < 0.001$). The infants born in autumn or winter were at an increased risk of developing anemia ($\chi^2 = 22.949$, $P < 0.001$). Birth weight had no effect on the rate of anemia in infants ($\chi^2 = 0.023$, $P = 0.568$). Infants who were exclusively breastfeeding had higher anemia rates than infants who were fed formula ($\chi^2 = 38.466$, $P < 0.001$). Infants whose caregivers added no complementary foods had higher anemia rates (24.7%) than infants whose caregivers added two or more types of complementary food (8.2%). The type of caregiver had no effect on infant anemia rates ($\chi^2 = 0.031$, $P = 1.000$). **Table 5** shows the multivariate logistic regression analysis results of the

TABLE 3 | Infant feeding practices.

Feeding practice	*n*	Percent (%)	Hemoglobin (g/L)
Feeding style			
Exclusive breastfeeding	634	56.3%	116.6 ± 8.5
Mixed feeding	296	26.3%	119.9 ± 7.0
Artificial feeding	197	17.5%	120.7 ± 7.3
Complementary foods			
None	85	3.7%	115.7 ± 11.7
One kind	390	32.3%	119.0 ± 8.9
More than two kinds	478	64.0%	119.1 ± 7.0
Caregivers			
Parents	202	41.6%	117.3 ± 7.6
Grandparents	253	52.1%	117.7 ± 8.2
Babysitters	31	6.4%	118.3 ± 9.7

risk factors for infant anemia. Gestational age at birth, birth season, feeding style and complementary food supplementation significantly affected infant anemia rates ($P < 0.05$).

As shown in **Table 6**, anemia had no significant effect on the DDST pass rates ($\chi^2 = 5.600$, $P = 0.051$).

DISCUSSION

The present study examined information associated with 1,127 6-months-old infants and revealed an anemia prevalence of 11.8%. In our study, the risk factors associated with anemia were gestational age at birth, birth season, feeding style and complementary food supplementation. No significant difference in the DDST pass rate was evident between infants with and without anemia.

The anemia rate in our study was lower than the global anemia rate (24.8%) (1). The WHO has estimated that anemia affects 1.62 billion people globally, including 293 million preschool-aged children, 56 million pregnant women and 468 million non-pregnant women. A total of 54.3% of infants aged 6–11 months are reportedly anemic, and 24.3% of infants in rural China suffer from moderate or severe anemia (20). Although the prevalence of anemia in China has gradually decreased, the adverse impacts of anemia on infants and society are profound. The most common form of anemia is iron deficiency anemia (21). Iron deficiency in children <3 years of age negatively affects their physical and intellectual development (22). Additionally, the prevalence of anemia is highest at the ages of 6–12 months, a period that is critical for psychomotor development. Some studies have shown that infants with iron deficiency have lower auditory brainstem response (ABR) responses than those with normal iron levels, representing the iron deficiency anemia infants with delayed central nervous system (CNS) myelination (23). Therefore, we aimed to identify the risk factors associated with anemia.

In our study, we found that premature infants had an increased risk of developing anemia at 6 months of age, which was consistent with other studies (24, 25). Halliday et al. (26) found that 26% of premature infants had iron deficiency during the first year of life. Preterm infants are at high risk of nutritional deficiency because they have low stores of iron, zinc and vitamin A (27). Preterm infants can experience blood loss at birth, inadequate erythropoiesis, blood sampling, rapid growth, hemorrhage and hemolysis. Therefore, most premature infants have smaller blood volumes and experience more profound anemia than full-term infants (28). The effects of iron deficiency include poor physical growth, gastrointestinal disturbances, neurodevelopmental impairments and altered immunity (29, 30). Therefore, premature infants should receive iron supplementation from sources, such as fortified human milk, iron-fortified formula or medicinal elemental iron (e.g., 2–4 mg/kg/d).

TABLE 4 | Factors associated with infant anemia.

Factors	Anemia *N* (%)	Non-anemia *N* (%)	χ^2	*P*
			1.799	0.196
Male	77 (13%)	514 (87%)		
Female	56 (10.4%)	480 (89.6%)		
Maternal educational level			2.132	0.352
Less than undergraduate	24 (13%)	161 (87%)		
Undergraduate	61 (10.5%)	519 (89.5%)		
More than undergraduate	39 (13.7%)	246 (86.3%)		
Maternal age			3.147	0.672[a]
<25 years	1 (12.5%)	7 (87.5%)		
25–29 years	38 (13.1%)	251 (86.9%)		
30–34 years	58 (10.4%)	498 (89.6%)		
35–39 years	22 (11.8%)	165 (88.2%)		
40–44 years	5 (15.6%)	27 (84.4%)		
>44 years	0 (0%)	1 (100%)		
Gestational age at birth			40.103	0.000*
<37 weeks	25 (38.5%)	40 (61.5%)		
>37 weeks	108 (10.2%)	954 (89.8%)		
Birth season			22.949	0.000*
Spring	13 (5.9%)	207 (94.1%)		
Summer	22 (7.5%)	270 (92.5%)		
Autumn	51 (15.2%)	285 (84.8%)		
Winter	47 (16.8%)	232 (83.2%)		
Birth weight			0.023	0.568
<2,500 g	4 (11.1%)	32 (88.9%)		
>2,500 g	127 (11.9%)	936 (88.1%)		
Feeding style			38.466	0.000*
Exclusive breastfeeding	108 (17%)	526 (83%)		
Mixed feeding	13 (4.4%)	283 (95.6%)		
Artificial feeding	12 (6.1%)	185 (93.9%)		
Complementary foods			21.509	0.000*
None	21 (24.7%)	64 (75.3%)		
One kind	51 (13.1%)	339 (86.9%)		
More than two kinds	44 (8.2%)	495 (91.8%)		
Caregivers			0.031	1.000
Parents	27 (13.4%)	175 (86.6%)		
Grandparents	34 (13.4%)	219 (86.6%)		
Babysitters	4 (12.9%)	27 (87.1%)		

[a] *Fisher's exact test.*
*$P < 0.001$.

TABLE 5 | Univariate analysis of factors influencing infant anemia.

Factors	*B*	SE	*P*	OR	95% CI for Exp (B)
Gestational age at birth	−1.979	0.303	0.000*	0.138	0.076–0.250
Birth season	−0.473	0.098	0.000*	0.623	0.515–0.755
Feeding style	0.847	0.169	0.000*	2.332	1.675–3.247
Complementary foods	0.254	0.096	0.008*	1.289	1.068–1.554

B, coefficient; SE, standard error; OR, odds ratio, which equals to the power of the coefficient B; 95% CI for Exp (B), 95% confidence interval of the exponentiation of the coefficient B.
**P < 0.001.*

TABLE 6 | Effect of anemia on DDST pass rates.

	Pass	Suspect	Abnormal	χ^2	*P*
Anemia	121 (91.0%)	8 (6.0%)	4 (3.0%)	5.600	0.051
Non-anemia	910 (91.5%)	77 (7.7%)	7 (0.7%)		

We showed that infants born in spring had lower Hb levels than infants born in winter. A previous study also showed that the incidence of anemia in infants aged 5–7 months who were born in spring and summer was higher than that in infants aged 5–7 months who were born in autumn or winter (31). This difference is probably due to seasonal variations in the folate and vitamin B6 statuses among women who may be attempting to become pregnant (32, 33).

In our study, we found that feeding style and complementary foods affected the prevalence of anemia in infants. Infants who were exclusively breastfeeding (17%) had a higher prevalence of anemia than infants whose diets were mixed or infants who were fed formula (4.4 and 6.1%). The formula consumed by the infants contained iron; therefore, the infants whose diets were mixed and the infants who were fed formula were unlikely to develop anemia. The addition of two or more types of complementary foods was associated with the lowest prevalence of anemia among the three groups (8.2 vs. 24.7% and 13.1%). The WHO recommends exclusive breastfeeding without the introduction of any nutritious complementary foods for the first 6 months (34). The concentration of iron in human milk is relatively low. In China, some parents do not add complementary foods or iron supplementation during the first 6 months, which has become an important cause of infant anemia. As the infant grows, iron from human milk becomes insufficient to meet the increasing needs of the body tissue and circulation (35). Therefore, complementary foods containing iron should be given to infants at the proper time to avoid anemia (36). In our study, the infants who were fed one type of food were always fed rice flour, and the infants who were fed two or more types of complementary foods were always fed liver paste, yolk or meat paste, which contain high levels of iron. Wang et al. (37) found that the introduction of complementary foods comprising rice cereal, porridge, and bread was more likely to result in the development of anemia than the introduction of animal-based foods. Rice cereal, porridge, and bread contain low amounts of bioavailable iron and have phytates that inhibit iron absorption (38). One study showed that deficiencies in vitamin A, vitamin C, zinc and iron were associated with the late introduction of complementary food (39). Thus, the addition of complementary foods should begin when maternal milk no longer meets the nutritional needs of the infant. Complementary foods not only provide nutrition to infants but also shape their future eating habits (40). However, some studies have shown that the early introduction of complementary foods is associated with allergies (41). Conversely, the late introduction of complementary foods is associated with developmental delays, such as motor skill deficits (42). In subsequent studies, we intend to identify a suitable time for the introduction of complementary foods.

In our study, we found that anemia was not associated with the DDST pass rates. This lack of association was probably because the duration of anemia in the infants was not sufficient to influence the DDST results. Iron requirements are most likely to exceed iron intake during the first 6–18 months of life because infants' growth and blood volume expansion proceed rapidly during this time (43). In our study, we investigated 6-months-old infants with anemia; thus, anemia had not been present for long. Lozoff et al. (44) showed that infants with iron deficiency anemia processed information slower at 12 months of age than infants with a good iron status. Infants with iron deficiency anemia have reduced dopamine function at 9 months, and this condition worsens at 12 months (45). Shafir et al. (46) found that 12- to 23-months-old infants with iron deficiency anemia did not catch up in motor development, although iron therapy during infancy corrected their anemia. In summary, anemia in infants should be detected as soon as possible. We examined Hb levels at the age of 6 months to select anemic infants and provide therapy early, thus preventing unfavorable outcomes caused by iron deficiency. Some studies have shown that at 6–8 weeks after birth, infants should receive iron supplementation (2–3 mg/kg per day) or formula containing iron (12 mg/L) to prevent iron deficiency anemia. Infants with birth weights below 1,000 g require additional iron (47).

Our study showed that the gestational age at birth, birth season, feeding style and complementary food supplementation affected anemia in infants aged 6 months. Early detection is of utmost importance to prevent adverse outcomes caused by infant anemia. Next, the caregiver should add iron-containing complementary foods, such as liver paste, yolk and meat paste, at a suitable time, especially in infants whose gestational age at birth is <37 weeks. In addition, infants born during different seasons should be supplied with nutrition accordingly. For example, infants born during spring should be provided with more iron than those born during winter.

Nevertheless, our study had several limitations. First, China is an expansive country that contains individuals of different ethnicities who inhabit different geographic locations and have various dietary traditions. These factors are probably associated with the prevalence of anemia. However, our study was limited to the population in Beijing. Further studies should focus on low-income and middle-income provinces in China. Second, we also lacked information regarding maternal anemia. Maternal anemia

has been associated with infant anemia (48). In future studies, we will examine data on maternal anemia.

CONCLUSIONS

Anemia is a global public health problem that influences infant development, resulting in poor outcomes in adulthood. The risk factors identified in our study, such as a gestational age at birth of <37 weeks, exclusive breastfeeding, a lack of supplementation with complementary foods and a spring birth date, may be meaningful for the early detection of infant anemia and the prompt delivery of interventions.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The study was carried out in accordance with the recommendations of the Clinical Research Ethics Committee of Peking University First Hospital. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of Peking University First Hospital, China.

AUTHOR CONTRIBUTIONS

QL conducted the experiments, analyzed the data, wrote the manuscript, and approved the final version to be published. YH contributed to the conception and design of the experiment, acquired the data, revised the manuscript, approved the final version to be published, and agreed to be accountable for all aspects of the work. FL and WL contributed to the conception and design of the experiment, acquired the data, critically revised the manuscript, and approved the final version to be published. WS contributed to the conception and design of the experiment and approved the final version to be published.

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Lactobacillus reuteri DSM 17938 Probiotics May Increase CC-Chemokine Receptor 7 Expression in Infants Treated With for Colic

Francesco Savino[1]*, *Ilaria Galliano*[2], *Andrea Savino*[2], *Valentina Daprà*[2], *Paola Montanari*[2], *Cristina Calvi*[2] and *Massimiliano Bergallo*[2]

[1] Department of Paediatrics, Azienda Ospedaliera Universitaria Città della Salute e della Scienza di Torino, Turin, Italy,
[2] Department of Public Health and Paediatric Sciences, Scuola di Medicina, Università degli Studi di Torino, Turin, Italy

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***Correspondence:**
Francesco Savino
francesco.savino@unito.it

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Aim: Studies have shown that *Lactobacilli reute*ri probiotics can affect cells that play a key role in the immune system. This *in vivo* Italian study investigated how *Lactobacillus reuteri* DSM 17938 influenced CC-chemokine receptor 7 (CCR7) and interleukin 10 (IL-10) in breastfed colicky infants.

Methods: Our University hospital in Turin recruited 50 healthy outpatients, at a median age of approximately 1 month, from September 2017 to August 2018. They were randomized to daily *Lactobacillus reuteri* DSM17938 (1×10^8 cfu) or a placebo for 28 days from recruitment. We collected peripheral blood and evaluated the expression of CCR7 messenger ribonucleic acid using the real-time TaqMan reverse transcription polymerase chain reaction method at baseline and after the study period.

Results: We found increased expression of CC-chemokine receptor 7 in infants treated with the probiotic, but not the controls ($p < 0.0026$). No differences were observed for interleukin 10 after the study period in either group. At baseline, daily crying time was comparable in the probiotic and control groups: 341 (25) vs. 337 (29) min., respectively ($p = 0.450$). After 28 days, daily mean crying time decrease statistically in the probiotic group: 78 (23) vs. 232 (31), respectively ($p < 0.001$).

Conclusion: The increase in CC-chemokine receptor 7 might have been a response to probiotic treatment. As a relatively small sample was used to conduct this study, our research needs to be replicated in different settings, and over time, to produce comparable findings.

Keywords: breastfeeding, CC-chemokine receptor 7, colicky infants, *Lactobacillus reuteri*, Interleukin 10

KEY NOTES

- *Lactobacilli reuteri* can affect cells that play a key role in the immune system.
- This *in vivo* study investigated how *Lactobacillus reuteri* DSM 17938 influenced CC-chemokine receptor 7 (CCR7) and interleukin 10 (IL-10) in 50 colicky breastfed infants.
- We found increased expression of CC-chemokine receptor 7 in infants randomized to the probiotic, but not placebo, group and no differences in interleukin 10 in either group.

INTRODUCTION

Inflammatory mediators, such as cytokines, chemokines and chemokines receptors, have been linked to immune system alterations and they display a considerable amount of similarity of function in infants (1). However, their precise mechanisms in early life are not fully understood.

Historically, neonates were assumed to be deficient in regulatory T cells and other adaptive immune cells, but further research has been carried out in this area in the last decade (2).

Chemokine receptors are involved in organizing thymic architecture and function and lymph-node homing of naive and regulatory T cells. They also influence inflammation, particularly chemokine receptor type 7 (CCR7), which is essential for the directed migration of adaptive immune cells, and regulatory T cell generation (3). The CCR7 signaling system has been implicated in diverse biological processes, such as lymph node homeostasis, T cell activation, immune tolerance and inflammatory responses (3)

CCR7 is a member of the G protein-coupled receptor family (syn CD197). It is activated by two different ligands, chemokine ligand 19 and chemokine ligand 21, and is involved in the migration, activation and survival of multiple cell types, including dendritic cells, T cells, eosinophils, B cells and endothelial cells (4).

Interleukin-10 (IL-10) is arguably the most potent anti-inflammatory cytokine and it is produced by nearly all of the innate and adaptive immune cells. IL-10 plays an important role in the maintenance of gut homeostasis, due to its anti-inflammatory functions. During an infection, IL-10 inhibits the activity of type 1 T helper cells, natural killer cells, and macrophages, which are required for optimal pathogen clearance, and contribute to tissue damage (5).

In infants, the mucosal immune system is constantly exposed to a wide range of commensal and potentially pathogenic microbial species. Intestinal intraepithelial lymphocytes provide a first line of protection and investigating their role in immunity is critical (6).

Emerging evidence supports the concept that infant colic could represent gut inflammation and microbial dysbiosis (7, 8). Lactobacilli are widely used as probiotics and have been shown to have beneficial effects on diarrhea that is associated with infections (9). However, they have also been used in clinical trials that have examined necrotising enterocolitis (10), inflammatory bowel diseases, and infantile colic (11).

Probiotics provide beneficial bacteria and clinicians use them because they can improve modifications in the intestinal microbiota and influence the immunological status of the host (9).

The beneficial effect of probiotics are mediated by several mechanisms, such as immune receptor cascade signaling and cytokines and toll like receptors (10) modulating inflammation and gene networks regulating production of bacterial-derived immunoregulatory molecules (12).

In a study published in 2018 we reported that infants with colic who were treated with *Lactobacilli reuteri* DSM17938 for 30 days demonstrated a significant decrease in calprotectin values and crying time. The infants also showed increased forkhead box P3 concentrations and these resulted in a decreased retinoic acid-related orphan receptor T and forkhead box P3 ratio (13), while the expression of mRNA expression of Toll like receptor 2 and Toll like receptor 4 seem not influenced (14). An *in vitro* study Cervantes -Barragan reported that molecular mechanisms responded to the inflammatory status of the gut during treatment with *Lactobacillus reuteri* (15).

One of these regulatory molecules is the IL-10, which is an anti-inflammatory cytokine and its effects have been demonstrated in studies in both mice and humans (14). Indeed, IL-10 signaling is essential for intestinal homeostasis, particularly in macrophages. IL-10 dampens intestinal inflammation and it would be interesting to know if it can be modulated by probiotic treatment (16, 17).

The aim of the present study was to investigate CCR7 and IL-10 in breastfed colicky infants treated with *Lactobacillus reuteri* DSM 17938 for 28 days.

PATIENTS AND METHODS

Subjects and Methods

This study was carried out at the Department of Pediatrics, Regina Margherita Children Hospital, Turin, Italy, between September 2017 and August 2018. We recruited 50 full-term exclusively breastfed infants aged <50 days and randomized equal numbers to receive either *Lactobacillus reuteri* DSM 17938 of a placebo for 28 days. The median age of the probiotic group at recruitment was 28.5 days and it was 32.5 days for the placebo group. They were included if they were born at a gestational age of between 37 and 40 weeks, with a birth weight of between 2,500 and 4,000 g and a 5-min Apgar score of more than seven. All the infants had colic and underwent blood tests during routine outpatient examinations. We used the modified Wessel's criteria for the diagnosis of infantile colic (13).

The study protocol was approved by the local Ethical Committee at Ospedale Mauriziano—Ospedale Infantile Regina Margherita—S. Anna Torino, and the infants' parents provided written consent to participate in the study.

Sample Collection

Venous blood samples were collected from the infants at 8 a.m., after a 3 h fasting period, which coincided with routine clinical blood sampling to minimize the disturbance to the infants. A

Abbreviations: CCR7, chemokine receptor type 7; IL10, interleukin 10; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; PCR, polymerase chain reaction.

tube of haemachrome was collected at recruitment, namely day 1 of the study period, for each infant and at the control visit after 28 days. Each sample was transferred to sterile Eppendorf tubes (Merck KGaA, Darmstadt, Germany) and stored in a freezer at −80°C until they were needed.

Intervention

The infants were randomized to receive the probiotic *Lactobacilli reuteri* DSM 17938 or placebo daily for 28 days using a computer programme. The active study product consisted of a suspension of freeze-dried *Lactobacilli reuteri* DSM 17938 in a mixture of sunflower oil and a medium-chain triglyceride oil, which was supplied in a 5 ml dark bottle fitted with a dropper cap. Five drops of the formulation delivered the daily dose of *Lactobacilli reuteri* 1×10^8 colony forming units and this was given 30 min before feeding. Both the *Lactobacilli reuteri* and placebo study products were provided by the Italian distributor (Nòos Srl, Roma, Italy). This oil suspension is stable for 21 months at 2°C to 8°C, as documented by the manufacturer (BioGaia AB, Stockholm. Sweden). During the study, parents were instructed to keep the product in the refrigerator when it was not in use.

Subjects received the study product free of charge after enrolment. Each child's pediatrician was informed, in writing, of the child's participation and parents were given a phone number so that they could ask the research team questions and report any perceived problems or concerns during the study.

Follow-Up Phase

Each participant was given a physical examination at enrolment, on day 1 and on day 28.

The parents completed a daily questionnaire for the duration of the study, which included the following data on the general health status of the infant, namely the occurrence of any illness and the use of antibiotics or other medication. It also included gastrointestinal signs and related symptoms, such as crying time, vomiting and regurgitation, and stooling habits.

Participants flow through study are reported in **Figure 1**.

Ribonucleic Acid (RNA) Extraction

Total RNA was extracted from 200 μl of blood using a Maxwell automated extractor (Promega, Wisconsin, USA) and the simplyRNA Blood Kit protocol (Promega) without modification. One microgram of total RNA was reverse-transcribed with 8 μl of 10X buffer, 4.8 μl of 25 mM $MgCl_2$, 2 μl of ImpromII (Promega), 1 μl of 40 U/l RNase inhibitor, 0.4 μl of 250 μM random hexamers (Promega), 2 μl of dNTP mix at 100 mM each (Promega) and double distilled water to create a final volume of 20 μl. The reaction was carried out using a GeneAmp PCR system 9700 Thermal Cycler (Applied Biosystems, California, USA) using the following conditions: 5 min at 25°C, 60 min at 42°C and 15 min at 70°C for enzyme inactivation. The complementary deoxyribonucleic acid (DNA) was stored at −80° until use.

Relative Quantification by Real-Time Polymerase Chain Reaction (PCR)

Relative quantification of the messenger RNA expression levels of the selected genes was achieved using TaqMan amplification (Lifetechnologies, Texas, USA) and normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene. This was achieved using an ABI PRISM 7500 real-time system (Life Technologies, Texas, USA). The mRNA messenger RNA expression levels of CC R7 and IL10 were quantified using real-time PCR. Approximately 100 ng of cDNAcomplementary DNA was amplified in a total volume of 20 μl containing 2× GoTaq® qPCR Master Mix (Promega, Madison, WI), 500 nmol of specific primers and 200 nmol of probe. Were used: CCR7 primers (CCR7F-5′- GCAACTCAACATCGCCTACG−3′)(CCR7R-5′-GAAGAGATCGTTGCGGAACTT−3′) and probe (CCR7P-6FAM- acccttcttgtacgccttcatcgg -TAMRA); IL10 primers (IL10F-5′- ATGAAGGATCAGCTGGACAACTT−3′) (IL10R-5′-CCTTGATGTCTGGGTCTTGGT-3′) and probe (IL10P-6FAM-ACCTGGGTTGCCAAGCCTTGTCTG -TAMRA); GAPDH primers (GAPDHF-5′-CCAAGGTCATCCATGACAAC-3′) (GAPDHR-5′- GTGGCAGTGATGGCATGGAC-3′) and probe (GAPDH-6FAM- TGGTATCGTGGAAGGA-3′ MGB). The amplifications were performed in a 96-well plate at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was run in triplicate. The target gene relative expression in patients was compared with normal samples using the 2-ΔΔCt method, and the relative expression is expressed in arbitrary units (AU).

The messenger RNA expression levels of CCR7 and IL10 and GAPDH were quantified by real-time PCR, as previously described by Mareschi et al. (18).

Statistical Analysis

The sample size was calculated on the basis that we wanted to find a 50 min reduction in daily average crying time in the probiotic group, when it was compared to the placebo group. This was considered a clinically relevant difference and was based on previous studies. We calculated that 20 patients were needed in each group, based on an alpha value of 0.05, beta value of 0.20 and an estimated standard deviation (SD) within the groups of 50 min crying time. We decided to enroll 25 subjects per group to allow for a 20% drop-out rate.

Randomization was performed by the random-digit method, using computer-generated numbers. We used a two-treatment randomization scheme with random blocks of varying size, employing Stata Statistical Software, Release 9 (StataCorp LP, College Station, Texas, USA) and the ralloc procedure. Data were analyzed by SPSS, version 16 (SPSS Inc, Illinois, USA), while the sample size calculation was performed by NCSS-PASS 2000 (Number Cruncher Statistical Systems, Utah, USA).

Data are shown as means and standard deviations (SD) or medians and interquartile ranges (IQR) for continuous variables, as appropriate, and numbers and percentages for categorical variables. Differences between the groups were evaluated using the Student's *t*-test for paired samples, while associations between the categorical variables were evaluated by Fisher's exact test. The Wilcoxon signed-rank test and Friedman test were used to evaluate differences between paired samples for continuous variables, when appropriate.

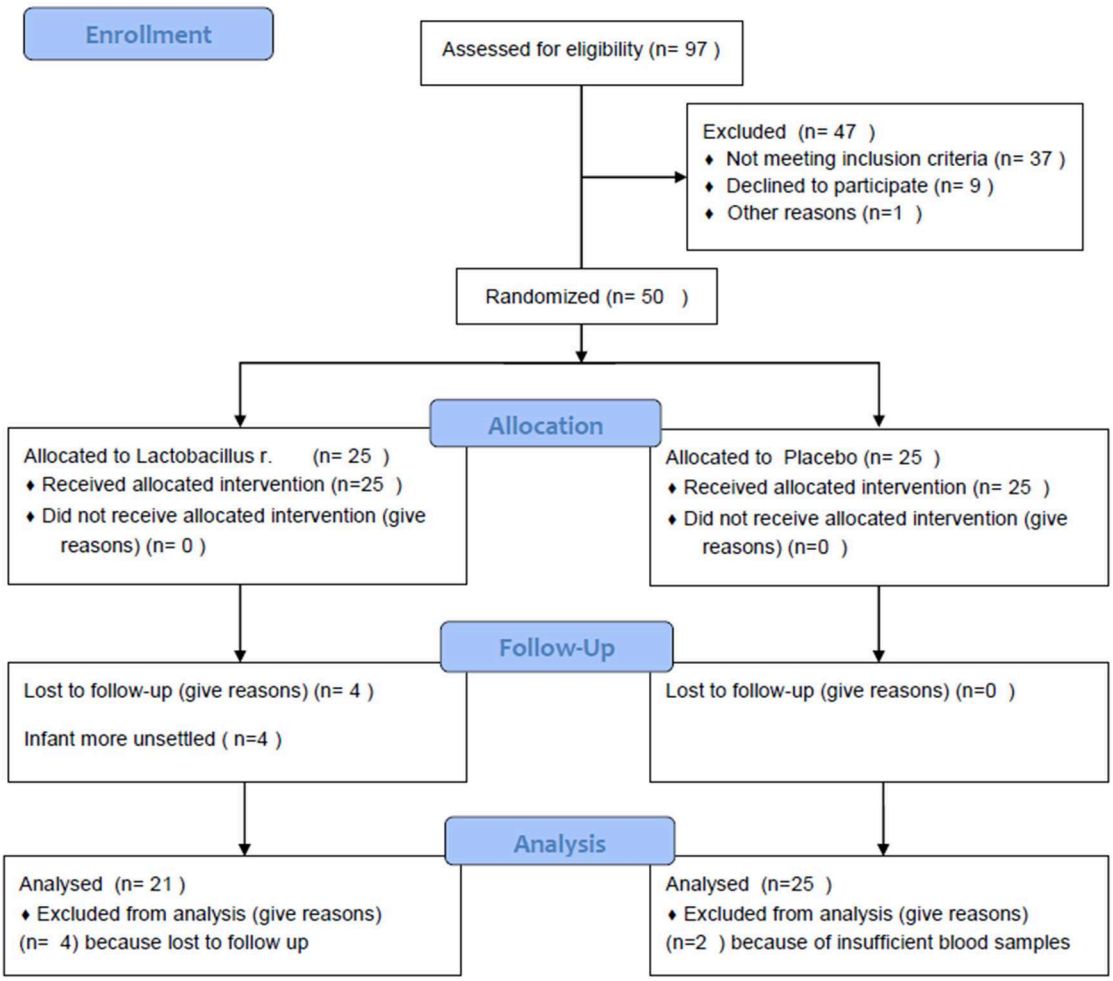

FIGURE 1 | Flow diagram.

All reported p-values were two-sided and differences were considered to be significant when $p < 0.05$.

RESULTS

A total of 50 infants were enrolled to the study and allocated, according to the protocol, to receive the *Lactobacilli reuteri* DSM17938 probiotic or placebo. The characteristics of the subjects enrolled and treated with the probiotic and the placebo are reported in **Table 1**. There were no significant differences between groups at baseline (**Table 1**). We excluded six infants from the analysis because of sufficient blood samples: four in the probiotic group and two in the placebo group.

The primary outcome was the measurement of the expression level of CC-chemokine receptor 7 messenger RNA in the study group at day 1 and at day 28.

The CC-chemokine receptor 7 values in the probiotic group on day 1 was 2.73 ± 1.1 ΔCt and on day 28 it was 1.82±1.45 ΔCt. In comparison, the CC-chemokine receptor 7 values in the placebo group was 1.86±1.1 ΔCt on day 1 and 1.21±0.6 ΔCt on day 28 (**Table 2**).

After the study period CC-chemokine receptor 7 were expressed at higher levels in the probiotic group than in the placebo group ($p = 0.0020$) (**Figure 2**).

When we analyzed the placebo group we did not observe different CCR7 expression on day 1 and on day 28 ($p = 0.26$) (data not shown).

Secondary Outcome

The values of IL-10 in the probiotic group were 8.64 ± 1.32 ΔCt on day 1, and 8.84 ± 2.31 ΔCt on day 28 ($p = 0.6552$)(**Figure 2**). The values in the placebo group were 8.61 ± 1.40 ΔCt on day 1 and 9.31 ± 1.30 ΔCt on day 28 ($p = 0.62$) (data not shown).

TABLE 1 | Baseline characteristics of the participants in the two study groups.

Variables	Probiotic ($n = 25$)	Placebo ($n = 25$)	p-value
Type of delivery (Cesarean), *n* (%)	6 (25)	13 (52)	0.079#
Male, *n* (%)	14 (56)	15 (60)	1.000#
Age at entry, median days (interquartile range)	28.5 (21)	32.5 (21)	0.382¶
Family history of GI diseases (yes), *n* (%)	8 (32)	6 (24)	0.754#
Family history of atopy (yes), *n* (%)	9 (36)	12 (48)	0.567#
Gestational age (weeks)	37.8 ± 0.5	37.9 ± 0.4	0.286*

Data are presented as means and standard deviations (SD).
*#Fisher's exact test; ¶Mann-Whitney test; *Student's t-test.*

TABLE 2 | Blood inflammatory markers (ΔCt), in infant with colic at enrolment and after 28 days of supplementation with *L. reuteri* or Placebo.

Variables	Probiotic ($n = 25$)	Placebo ($n = 25$)	p-value
IL 10 (ΔCt) DS (day 1)	8.64 ± 1.10	8.61 ± 1.40	0.111¶
IL 10 (ΔCt) DS (day 28)	8.84 ± 2.31	9.31 ± 1.30	
CCR7 (ΔCt) DS (day 1)	2.73 ± 1.10	1.86 ± 1.10	0.86¶
CCR7 (ΔCt) DS (day 28)	1.82 ± 1.45	1.21 ± 0.60	

Data are presented as means and standard deviations (SD).
¶Mann-Whitney test.

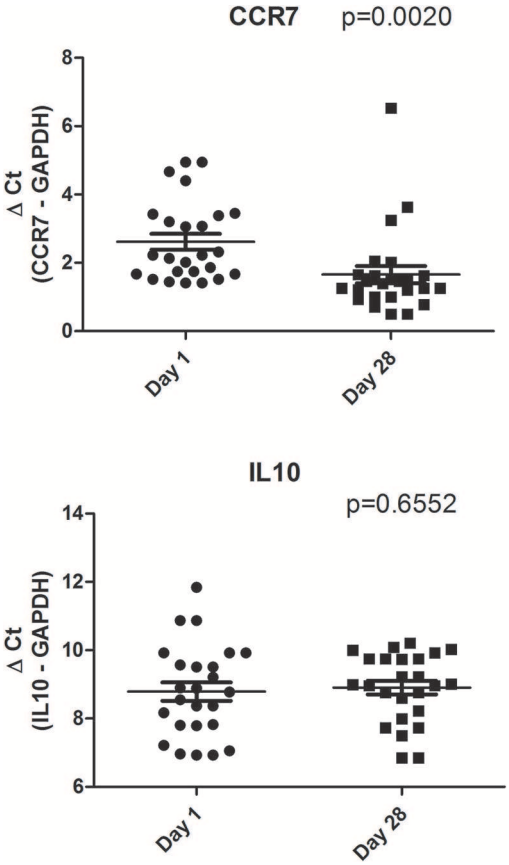

FIGURE 2 | CCR7 and IL10 transcriptional levels in the venous blood of group treated with L Reuteri DSM 17938. Data are represented as circle plots (day 1) and box plots (day 28), with horizontal lines depicting the averages. Relative messenger RNA levels were analyzed by real-time PCR and represented by ΔCt. The Paired *t*-test was performed.

At day 28, IL10 was equally expressed in the probiotic (**Figure 2**) and control groups ($p = 0.4375$) (data not shown).

Crying time of infants treated with *L. reuteri* or Placebo are reported in **Table 3**.

After *L. reuteri* administration for 28 days in infants with colic, we observed a significant decrease of daily crying time (341.55 ± 25.80 min/day at day 0 vs. 78.34 ± 23.24 min/day at day 28, $p = 0.001$) (**Table 3**).

We conducted on response variable (crying time) an ITT (Intention to Treat) analysis too, including 4 infants dropped out. As they were all from the Placebo group we classified them as responders.

Infants Responders (with a reduction of 50% in crying time from baseline) were significantly higher in the *L. reuteri* group vs. placebo group on days 28 (21 vs. 11, $p = 0.007$).

TABLE 3 | Crying and fussing time (mean minutes per days) at day 1 and at day 28 in placebo and *L. reuteri* group.

Variable (minutes)	Placebo ($n = 25$)	*L. reuteri* ($n = 25$)	*P*-value
Crying time per day 1	337.21 ± 29.2	341.55 ± 25.80	N.S.
Crying time per day 28	232.24 ± 31	78.34 ± 23.24	<0.001¶

Data as mean ± standard deviation.
Statistical analysis: ¶Mann-Whitney test.

DISCUSSION

The role of the main cytokines, chemokines and their receptors in the pathophysiology of disorders that involve inflammation in an emerging issue of active research (15, 16, 19–22).

Gut microbiota and its metabolites have been shown to influence immune functions and immune homeostasis within the gut, particularly during the first few months of life. In addition, probiotics have been shown to have an effect on immunity throughout cytokines and the production of metabolites such as tryptophan (15). Haileselassie et al. reported that *Lactobacillus reut*eri was able to influence the generation of monocyte-derived dendritic cells and subsequent autologous T cell responses (23).

On the other hand an *in vitro* study by Cervantes et al. demonstrated that *Lactobacillus reuteri* induced gut intraepithelial CD4(+)CD8αα(+) T cells (15).

The immunological mechanism behind the effects of probiotic such as *Lactobacillus reuteri* are probably regulatory T cells cell, as previous studies have reported (13, 14, 22, 23).

In fact Yuying et al. showed that *Lactobacillus reuteri* DSM 17938 changed the frequency of forkhead box P3 ratio regulatory T cells in the intestine and mesenteric lymph node in experimental necrotising enterocolitis (24).

While the precise mechanism is unclear, we can argue that the reaction between *Lactobacillus reuteri* and both epithelial and non-epithelial enteric cells must be active in modulating intrinsic anti-inflammatory effects in the intestine, as shown in animal models *in vivo* (23–27).

In this study we showed that *Lactobacillus. reuteri* 17938 was effective in alleviation of crying time due to colic in breast fed infants. Our results are consistent with the recent meta-analysis of previous four randomized trials (11).

Our findings showed also an increased expression of CC-chemokine receptor 7 in infants treated with *Lactobacilli reuteri* DSM 17938 after 28 days ($p = < 0.0020$), compared to day 1, while no differences where observed for IL-10 values.

CCR7 is involved in efficient induction of immune reactions as well as their silencing and regulation. Most of the knowledge on the involvement of CCR7 in the development of immunity and tolerance has been derived from mouse models and data on CCR7 function in humans is rather sparse (3, 4). However, it has been discovered that CCR7 contributes to the induction and maintenance of tolerance and this suggests a possible new strategy for treatment using probiotics.

CCR7 has recently been shown to be up-regulated in peripheral blood DCs and in DCs derived from monocytes and CD34 progenitor cells after activation with a variety of agents (28).

Our *in vivo* results using peripheral blood mononuclear cells from treated infants with L reuteri showed up-regulation of CCR7 expression levels. that the baseline levels of CCR7 were low.

These results, preliminary in nature, are consistent with the hypothesis that level of expression of CCR7 maybe the response to recruitment to gut lymphatics systems.

While our data suggest that IL-10 cytokines seem not be involved by probiotic supplementation, but a larger sample is necessary to confirm our observations, which were echoed by an animal study carried out by Yan et al. (29).

Strength and Limitations

There were some limitations to our study. First, since this was an experimental study, it only provides preliminary data. It is difficult to obtain detailed information on many factors when carrying out a pilot study with a small number of subjects. That is why it is necessary to control for the effects of extraneous variables that might result in misleading interpretations of causality. As a result, the findings of this study cannot be generalized.

However, to the best of our knowledge this is the first research study that investigated CCR7 in healthy breastfed infants treated with *Lactobacillus reuteri* DSM 17938 compared to a placebo using real time PCR. Tompa et al. reported that cryopreservation of peripheral blood mononuclear cells could have an impact on the expression of CCR7 on regulatory T cells, but we did not use cytofluorimetry and our data were not influenced by storage (30).

The standardized approach that we used permits the study to be replicated in different settings, or over time, to produce comparable findings.

CONCLUSION

Our randomized study of breastfed infants with colic found a decreased crying time and an increased expression of CC-chemokine receptor 7 in infants treated with the probiotic *Lactobacillus reuteri* DSM 17938 for 28 days, but not the placebo group. No differences were observed for interleukin 10 after the study period in either group. The increase in CC-chemokine receptor 7 might have been a response to probiotic treatment.

Understanding the possible effect of probiotic supplements, particularly during the first few weeks of life, is interesting and could also help to induce oral tolerance. Future studies on CCR7 in human molecular medicine, as well as more refined microbiota models of site-specific and inducible CCR7 and the CCR7-ligand, will help our understanding of this important molecular pathway. It will also aid our understanding of the cellular responses driven by it during probiotic treatment. Taking advantage of these new features may lead to a tailored approach for regulatory T cells using probiotic treatment.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The study protocol was approved by the local Ethical Committee at Ospedale Mauriziano—Ospedale Infantile Regina Margherita–S. Anna Torino, and the infants' parents provided written consent to participate in the study.

AUTHOR CONTRIBUTIONS

FS had primary responsibility for protocol development as principal investigator and wrote the manuscript. IG performed PCR analysis and helped to write the manuscript. AS analyzed data and wrote the manuscript, edited references. VD performed PCR analysis and helped to write the manuscript. PM performed PCR analysis and helped to write the manuscript. CC performed analysis and wrote the manuscript. MB performed the final data analysis, supervised analysis and helped to write the manuscript.

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Human Milk Oligosaccharide Composition Is Associated With Excessive Weight Gain During Exclusive Breastfeeding—An Explorative Study

Melanie W. Larsson[1,2†], Mads V. Lind[1†], Rikke Pilmann Laursen[1], Chloe Yonemitsu[3], Anni Larnkjær[1], Christian Mølgaard[1], Kim F. Michaelsen[1] and Lars Bode[3]*

[1] Department of Nutrition, Exercise, and Sports, University of Copenhagen, København, Denmark, [2] Department of Nursing and Nutrition, University College Copenhagen, København, Denmark, [3] Department of Pediatrics and Larsson-Rosenquist Foundation Mother-Milk-Infant Center of Research Excellence, University of California, San Diego, La Jolla, CA, United States

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***Correspondence:**
Melanie W. Larsson
mela@kp.dk

[†]These authors share first authorship

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Background: Some infants experience excessive weight gain during exclusive breastfeeding. The cause is unknown, but variation in human milk composition might play a role. Several human milk koligosaccharides (HMOs) have been associated with growth velocity in breastfed infants, and it has been suggested that the mechanism could be through an effect on infant gut microbiota composition.

Objective: The purpose of this exploratory study was to evaluate if HMO composition was different in milk fed to infants with excessive weight gain compared to infants with normal weight gain. Furthermore, we aimed to examine if HMO composition was associated with growth velocity and change in body composition and if there were maternal determinants of HMO composition.

Materials and Methods: We recruited 13 high weight-gain (HW) and 17 normal weight-gain (NW) breastfed infants, collected human milk and anthropometry data at 5 and 9 months, and analyzed HMO composition by high performance liquid chromatography.

Results: In the HW group eight out of 11 infants received milk from secretor mothers and in the NW group 15 out of 17. Comparing milk from Secretor mothers only, four HMO's were significantly different between the HW and NW group at 5 months and two remained significant at 9 months. Total HMO concentrations as well as total HMO-bound fucose at 5 months were positively associated with both fat mass index (FMI) and weight velocity from 0 to 5 months (all $p < 0.025$). 2′-fucosyllactose (2′-FL) was positively associated with weight velocity from 0 to 5 months and FMI at 5 months. In contrast, lacto-N-neotetraose was lower in the HW group ($p = 0.012$) and negatively associated with height-for-age Z-scores ($p = 0.008$), weight velocity from 0 to 5 months ($p = 0.009$) and FMI ($p = 0.033$). Maternal BMI at 5 months was negatively associated with 6′-sialyllactose and sialyl-lacto-N-tetraose (LSTb) and positively with 2′-FL, total HMO and total HMO-bound fucose (all $p \leq 0.03$).

Conclusion: In a small cohort, we found significantly different HMO concentrations in milk to exclusively breastfed infants with excessive weight gain, suggesting that some HMOs, including 2′-FL, which is the most abundant HMO and currently added to some infant formula, could be part of the cause for the excessive weight gain.

Keywords: growth, obesity, infancy, breastfeeding, human milk, human milk oligosaccharides, infant feeding, infant

INTRODUCTION

Human milk is recommended as the optimal nutrition for infants due to a wide range of beneficial effects for both mother and infant (1). This includes a potential protective effect against later overweight and obesity for the child, even though not all studies support this view (1–3). The conflicting findings may be partially due to the diverse composition of human milk, which contains macronutrients, micronutrients, and a host of bioactive compounds, some of which seem to affect growth and body composition (4, 5). Human milk oligosaccharides (HMOs) have recently been linked to growth in early infancy. This was observed in 37 mother-infant dyads where an increase in lacto-N-fucopentaose (LNFP) I was associated with lower infant weight at 1 and at 6 months, and lower lean and fat mass at 6 months. Further, LNFP II and disialyl-lacto-N-tetraose (DSLNT) were associated with higher fat mass at 6 months (6). Adding HMOs to infant formula has also gained interest because of the potential positive effects on the microbiota and the immune system of the infant. An intervention study adding the HMOs 2′-fucosyllactose (2′-FL) and lacto-N-neotetraose (LNnT) to infant formula showed lower morbidity and no effect on growth (7). Little is known about which factors determine the variability in HMO concentration, however, single nucleotide polymorphisms in the fucosyltransferase 2 (FUT2) secretor gene result in human milk that is deficient in α1,2-fucosylated oligosaccharides. Women with an active FUT2 enzyme are referred to as Secretors as they secrete a substantial amount of α1,2-fucosylated oligosaccharides; women with an inactive FUT2 that lack α1,2-fucosylated oligosaccharides are referred to as Non-secretors.

Some exclusively breastfed infants have an excessive weight gain in the first 6 months of life, but it is not known if the risk of later obesity is lower in this group of infants compared to infants breastfed less. Only a few studies have targeted this group of infants and therefore little is known about the causes for this excessive weight gain (8–12). Since the high weight gain occurs during the exclusive breastfeeding period and some studies show a noticeable catch-down after introduction to complementary food (9–11), it is reasonable to search for answers for this growth pattern in human milk composition itself. We have recently shown that infants with excessive weight gain during exclusive breastfeeding in the SKOT III cohort had a marked catch-down in weight and BMI z-scores from 5 to 9 months, when complementary foods were introduced (11).

Since some HMOs are related to growth, we hypothesized that differences in HMO composition could be part of the explanation for the early excessive weight gain seen in some exclusively breastfed infants. To test this hypothesis, we analyzed HMO composition in milk samples from the exploratory SKOT III cohort of exclusively breastfed infants with excessive weight-gain and compared the values with a group of exclusively breastfed infants with normal weight-gain.

MATERIALS AND METHODS

Study Design and Subjects

The mother-infant dyads were part of an ongoing prospective observational cohort study, the SKOT III cohort. The cohort included mothers and their 4–6 months old infants in two groups based on weight-for-age z-scores (WAZ). Infants with a WAZ > 2 and an increment of $> +1$ SDS in WAZ during the first 5 months post-partum, were recruited to a high weight-gain group (HW-group); infants with a WAZ between -1.0 and $+1.0$ SD were recruited to a normal weight-gain (NW) group. Further inclusion criteria for both groups were exclusive or full breastfeeding to at least 4 months post-partum. Infants in both groups were examined at age 5–6½ months and 9 months ± 2 weeks. Recruitment and overall study design has previously been described in detail together with human milk intake, content of macronutrients and hormones, as well as infant growth and body composition in the two groups (11). We did not match the infants based on sex, birth weight, maternal age, parity, and mode or place of delivery, however, the groups were overall well-matched.

The study protocol was approved by the Regional Ethical Committee of the Capital Region of Denmark in accordance with the Helsinki declaration (H-15008948) and the Data Protection Agency (2015-57-0117 & 2015-57-0116) and written informed consent was obtained from all parents.

Seventeen infants were referred according to the WAZ inclusion criteria to the HW group. However, four infants were excluded since they did not experience excessive weight gain although they had a WAZ $>$ 2SDS at the first examination. Two infants were excluded because they had a high length and therefore a BAZ < 2.00 and two were excluded because they had a relatively low weight gain from birth to 5 months (their birthweights were above 4.00 kg and changes in WAZ from birth to 5 months were -0.16 and $+0.6$ and could therefore not be defined as excessive weight gain). Thus, the HW-group included 13 infants with at least + 1.0 SDS increment in WAZ during the first 5–6 months post-partum. In the NW-group 42 parents showed interest in participating. Of these, 19 mother-infant dyads fulfilled the inclusion criteria. However, two infants were excluded. One had a low birth weight (2.67 kg; WAZ -1.3 SDS) followed by a catch-up with a WAZ increment of 1.8 SDS.

The other one had a birth weight of 4.66 kg (WAZ +2.5 SDS) followed by a catch down in WAZ of−2.9 SDS. Thus, the NW-group included 17 infants with an increment in WAZ during the first 5–6 months post-partum within normal range, defined as <0.67 SDS.

Anthropometry

Anthropometric measurements have been described in detail previously (11). In brief, weight and length were measured using standard procedures. Body composition was measured using Bioelectrical Impedance Analyzer (BIA) Quantum III (RJL Systems, Michigan, USA) and fat free mass (FFM), fat mass (FM), and fat mass percentage (FM%) were then calculated using the Lingwood Equation (11, 13). Maternal pre-pregnancy BMI as well as gestational weight gain were self-reported while maternal weight and height were measured using standardized procedure at the infant's age 5 months visit (11).

24 h Milk Volume

The 24 h milk intake has been explained in detail previously (11). To measure the 24 h milk intake at 5 months, mothers weighed their infants for a period of 72 h before and after each breastfeeding-session (each feed) using an electronic baby weighing scale (Tanita BD 815 MA, Tanita Corporation, Tokyo, Japan). Calculation of intake in grams was done by subtracting weight of the infant before the feed from the weight after the feed. In cases where test weighing was not completed for all feeds, the intake was estimated using an average of intake per feed calculated from the mother's registration. No correction for infant insensible water loss was made, and therefore the milk intake is likely to be underestimated by 3–10% (14, 15).

Human Milk Sampling and HMO Analysis

Mothers were asked to pump the entire content of both breasts using a manual breast pump (Type Harmony^{TM}, Medela AG, Baar, Switzerland) at infant age 5–6½ months and 9 months. The milk samples were stored at −20°C in the homes of the participants and transported in a bag with an ice pack and stored at −80°C at the University of Copenhagen. HMOs were analyzed in well-mixed samples of right and left breast at the University of California, San Diego, using high performance liquid chromatography (HPLC) after fluorescent derivatization (16). In brief, raffinose was added to the milk samples as an internal standard to allow for absolute HMO quantification. Oligosaccharides were isolated by solid phase extraction (SPE) over C18 and carbograph microcolumns, derivatized with 2-aminobenzamine (2AB), and further purified over silica gel SPE microcolumns. 2AB-labeled HMOs were analyzed by HPLC on an amide column with fluorescent detection. HMOs were annotated based on retention time and offline mass spectrometry and quantified based on standard response curves and in relation to the internal standard. Secretor status was determined based on presence or near-absence of 2′-FL and lacto-N-fucopentaose I. Since overall HMO composition differs between Secretors and Non-secretors, HMO were compared between the HW and NW for Secretors and Non-secretors combined as well as stratified by

TABLE 1 | Maternal and child characteristics according to high weight gain (HW) or normal weight gain (NW) group[a].

Infant Characteristics	HW group	NW group	*p*-value[b]
At birth			
N	13	17	
Gender (girls) n(%)	5 (38.5)	10 (58.8)	0.27
Gestational age, weeks	40.7 (40.5, 41.1)	40.4 (39.7, 41.3)	0.42
Cesarean delivery, n(%)	4 (33.3)	2 (11.8)	0.20
Weight, kg	3.99 ± 0.41	3.55 ± 0.31	**0.006**
Length, cm	53.0 ±1.4	51.7 ±1.7	**0.045**
Weight-for-age z-score	1.32 ± 0.75	0.54 ± 0.61	**0.006**
BMI-for-age z-score	0.56 ± 0.80	−0.08 ± 0.59	**0.025**
5 month visit			
N	13	17	
Age, months	5.6 ±0.5	5.9 ±0.3	0.054
Weight, kg	10.60 ±0.88	7.89 ±0.50	**<0.001**
Length, cm	70.5 ±2.2	68.1 ±2.1	**0.009**
Weight-for-age z-score	3.02 ± 0.76	0.39 ± 0.55	**<0.001**
BMI-for-age z-score	2.49 ± 0.99	−0.09 ± 0.83	**<0.001**
Fat mass percentage, %	35.40 ± 3.18	27.78 ± 3.14	**<0.001**
Weight velocity birth to 5 month visit, g/w	271.9 ±32.4	169.2 ±17.1	**<0.001**
WAZ change from birth to 5 month visit	1.71 ± 0.69	−0.15 ± 0.68	**<0.001**
24 h milk intake, g	981.9 (653.8–1321.4)	852.0 (482.7–1234.7)	0.19
9 month visit			
N	12	17	
Age, months	9.0 ±0.3	9.1 ±0.2	0.48
Weight, kg	11.65 ±0.79	9.03 ±0.37	**<0.001**
Length, cm	75.7 ±1.9	72.5 ±2.0	**<0.001**
Weight-for-age z-score	2.63 ± 0.56	0.47 ± 0.49	**<0.001**
BMI-for-age z-score	2.06 ± 0.89	0.15 ± 0.80	**<0.001**
Fat mass percentage, %	36.12 ± 3.50	31.02 ± 2.26	**<0.001**
Weight velocity 5 to 9 month visit, g/w	80.4 ±22.47	81.9 ±20.76	0.93
WAZ change from 5 to 9 month visit	−0.30 ± 0.34	0.08 ± 0.29	**0.004**
Maternal Characteristics			
Age, years	32.5 ±3.9	33.7 ±3.2	0.40
Parity, >1 (%)	53.9	52.9	0.96
Pre-pregnancy weight, kg	71.9 ±12.0	60.6 ±6.0	**0.002**
Height, cm	170.2 ±5.5	165.8 ±7.3	0.077
Pre-pregnancy BMI, kg/m^2	24.2 (22.1, 26.6)	22.2 (21.2, 22.5)	0.082
Gestational weight gain, kg	14 (12, 17)	15 (13, 18)	0.400
Weight, kg at 5 months	75.7 ±16.8	62.2 ±5.5	**0.004**
Weight, kg at 9 months	70.9 ±15.9	61.9 ±5.9	0.053

[a] *Values are expressed as mean ± standard deviation, median (25th, 75th percentile) or number (percentage) as appropriate. HW, high weight-gain group; NW, normal weight-gain group.*

[b] *p-values for differences between groups analyzed by Fisher's exact Chi-squared test for proportions, by independent t-test for age at visit, and by linear model adjusted for sex for all other variables. Bold values indicate significance at a P < 0.05 level.*

TABLE 2 | Human milk oligosaccharides content and 24 h intake according to high weight gain (HW) or normal weight gain (NW) groups, including only Secretors at 5 and 9 months[a].

HMOs	Concentration (nmol/ml)			Absolute intake (mg/d)		
	HW	NW	*p*-value	HW	NW	*p*-value
Secretor, *n* yes (%)						
5 mo	8 (72)	15 (87)				
9 mo	6 (75)	12 (86)				
Fucosylated or sialylated lactose						
2′-FL						
5 mo	8360 (7045, 10276)	6126 (5112, 7000)	0.061	3885 (2933, 5460)	2360 (1987, 3693)	0.096
9 mo	8201 (6269, 10962)	5191 (4228, 7598)	0.160			
3-FL						
5 mo	479 (422, 676)	470 (355, 577)	0.519	226 (187, 331)	191 (142, 298)	0.346
9 mo	709 (673, 773)	672.86 (400.55, 920.70)	0.851			
DFLac						
5 mo	888 (850, 1005)	781 (593, 868)	**0.045**	628 (421, 725)	389 (267, 567)	0.082
9 mo	1221.27 (945.75, 1587.07)	978.96 (769.49, 1358.50)	0.223			
3′-SL						
5 mo	896 (818, 1030)	777 (614, 1042)	0.401	556 (413, 717)	407 (318, 584)	0.218
9 mo	1104 (1004, 1373)	1125 (700, 1716)	0.851			
6′-SL						
5 mo	21 (193, 246)	247 (227, 315)	0.071	145 (116, 157)	128 (105, 163)	0.942
9 mo	102.51 (87.59, 205.27)	148.70 (116.79, 207.36)	0.512			
Non-fucosylated, non-sialylated HMOs						
LNT						
5 mo	1109 (768, 1475)	1340 (1064, 1511)	0.333	845 (637, 915)	700 (555, 922)	0.942
9 mo	598 (477, 669)	706 (502, 1344)	0.399			
LNnT						
5 mo	592 (525, 649)	817 (689, 974)	**0.012**	399 (330, 509)	438 (380, 620)	0.311
9 mo	424 (365, 543)	736 (504, 1061)	**0.049**			
LNH						
5 mo	84 (74, 96)	101 (76, 144)	0.175	76 (62, 108)	91 (80, 122)	0.772
9 mo	43 (41, 87)	43 (33, 62)	0.512			
Fucosylated, non-sialylated HMOs						
LNFP I						
5 mo	680 (552, 894)	932 (449, 1782)	0.846	585 (411, 708)	956 (286, 1184)	0.612
9 mo	723 (576, 786)	684 (470, 1258)	0.925			
LNFP II						
5 mo	1656 (1496, 2251)	1926 (1415, 2217)	0.796	1553 (1180, 1790)	1191 (909, 1696)	0.515
9 mo	1436 (1291, 1535)	1445 (1220, 2154)	0.708			
LNFP III						
5 mo	68 (59, 85)	91 (77, 132)	0.071	65 (47, 80)	65 (58, 83)	0.717
9 mo	82 (65, 96)	96 (84, 115)	0.160			
DFLNT						
5 mo	1565 (1410, 1601)	1486 (1422, 1720)	0.949	1354 (1140, 1622)	1213 (994, 1489)	0.247
9 mo	1863 (1531, 1992)	1827 (1590, 2054)	0.851			
FLNH						
5 mo	36 (26, 40)	39 (32, 69)	0.197	40 (31, 46)	40 (35, 59)	0.515
9 mo	16 (16, 19)	17 (10, 27)	1.000			
DFLNH						
5 mo	15 (13, 20)	25 (17, 50)	**0.045**	20 (18, 26)	36 (17, 54)	0.070
9 mo	14 (13, 18)	15 (12, 29)	0.640			

(Continued)

TABLE 2 | Continued

HMOs	Concentration (nmol/ml)			Absolute intake (mg/d)		
	HW	NW	*p*-value	HW	NW	*p*-value
Non-fucosylated, sialylated HMOs						
LSTb						
5 mo	111 (80, 159)	149 (115, 160)	0.333	111 (88, 132)	123 (80, 145)	0.612
9 mo	132 (70, 208)	158 (148, 182)	0.512			
LSTc						
5 mo	31 (12, 33)	33 (15, 42)	0.175	23 (11, 35)	26 (14, 31)	1.000
9 mo	12.67 (8.11, 17.41)	8.62 (6.29, 16.38)	0.851			
DSLNT						
5 mo	275 (213, 389)	311 (253, 375)	0.606	308 (265, 512)	293 (262, 481)	0.664
9 mo	413 (252, 553)	395 (372, 437)	0.851			
DSLNH						
5 mo	24 (16, 43)	37 (20, 52)	0.220	43 (26, 57)	59 (18, 71)	0.385
9 mo	4 (4, 7)	15 (8, 27)	0.190			
Fucosylated, sialylated HMOs						
FDSLNH						
5 mo	184 (135, 257)	326 (168, 404)	0.061	337 (223, 537)	503 (225, 586)	0.515
9 mo	139 (12, 158)	223 (147, 367)	0.111			
HMO-bound Fucose						
5 mo	16580 (15740, 17856)	14981 (14196, 15786)	**0.033**			
9 mo	18115 (16353, 19416)	14994 (14137, 17079)	**0.049**			
HMO-bound Sialic acid						
5 mo	2176 (1993, 2678)	2743 (2276, 2974)	0.366			
9 mo	2413 (2135, 2989)	2784 (2583, 3378)	0.261			
HMO sum						
5 mo	17602 (17022, 18474)	16584 (15656, 17199)	0.061	11039 (8707, 14336)	9832 (8054, 11430)	0.277
9 mo	17771 (16462, 19312)	15623 (15134, 17773)	0.061			
Diversity						
5 mo	3.92 (3.06, 4.67)	5.48 (4.85, 5.92)	0.061			
9 mo	4.10 (2.98, 5.19)	6.01 (4.39, 6.78)	0.160			

[a]*Data is presented for Secretors only as median (IQR) and tested using Mann-Whitney U-test. mo, months; 2′-FL, 2′-fucosyllactose; 3-FL, 3-fucosyllactose; 3′-SL, 3′-sialyllactose; 6′-SL, 6′-sialyllactose; LNT, lacto-N-tetraose; LNnT, lacto-N-neotetraose; LNFP I-II-III, lacto-N-fucopentaose; LSTb, LSTc, sialyl-lacto-N-tetraose; DSLNT, disialyl-lacto-N-tetraose; DFLac, difucosyl-lactose; DFLNT, difucosyl-lacto-N-tetraose; LNH, lacto-N-hexaose; FLNH, fucosyl-lacto-N-hexaose; DFLNH, difucosyl-lacto-N-hexaose; FDSLNH, fucosyl-disialyl-lacto-N-hexaose; DSLNH, disialyl-lacto-N-hexaose; HMOs, human milk oligosaccharides. Bold values indicate significance at a P < 0.05 level.*

secretor status. However, as there was only a very few infants in the Non-secretor group this was not examined separately. HMO concentrations per mL were multiplied with total 24 h milk intake volume to yield absolute HMO intake per 24 h. To assess the overall diversity of HMO composition, Simpson's Diversity index was calculated as the reciprocal sum of the square of the relative abundance of each HMO.

Statistical Analysis

Statistical analyses were performed using the R statistical environment (http://cran.r-project.org/, version 3.4.0). Descriptive results are presented as means and SD, mean and range or median and interquartile range (IQR: 25th and 75th percentile) as appropriate.

Differences between groups baseline characteristics was analyzed by Fisher's exact Chi-squared test for proportions, by independent *t*-test for age at visit, and by linear model adjusted for sex for all other variables. Mann-Whitney *U*-test was used to test differences between the two study groups, as HMO concentrations were not normally distributed. Associations between the different HMOs and infant WAZ, BMI-for-age z-scores (BAZ) and height-for-age z-scores (HAZ), fat mass index (FMI), fat free mass index (FFMI) and weight velocity from birth to 5 months as well as associations between mothers BMI, gestational weight gain and HMOs were investigated by Spearman's correlation, for the groups combined. We did not adjust for multiple confounders since this was not feasible given the small sample size. We used both raw and Benjamini-Hochberg false discovery rate–corrected *P*-values. In line with the explorative nature of the work we treated results with a raw $P < 0.05$ as being of interest. None of the false discovery rate–corrected *P*-values were <0.05 and we have only reported raw *p*-values.

RESULTS

Mother and Child Characteristics

Infants in the HW group weighed ∼450 g more and were 1.3 cm longer at birth than the NW group (**Table 1**). From birth to the 5 months visit weight gain was excessive in the HW group with about 100 g/week more than the NW group ($p < 0.001$). From the 5 to the 9 months visit weight gain per week was the same in the two groups (about 80 g/week, **Table 1**), but WAZ decreased in the HW group and was unchanged in the NW group. The BAZ decreased from 5 to 9 months from 2.49 to 2.06 in the HW group. The mothers in the two groups were of the same age and the same percentage was primipara (**Table 1**). The mothers in the HW group had higher body weight pre-pregnancy and at 5 months, but there was no difference in BMI either pre-pregnancy or at 5 months. The mean duration of exclusive or full breastfeeding for the HW and NW group, were 5.14 months (range: 3.69–6.46) and 5.54 months (range: 3.46–6.46), respectively ($p = 0.29$). At 9 months 83% of the infants in the HW and 94% in the NW group were still breastfed ($p = 0.56$).

Mean 24 h milk intake was 130 g (15%) higher in the HW group, but the difference was not significant (**Table 1**) and there was no difference in human milk intake per kg per day between the groups (data not shown). At the 5 months visit 73% of the infants in the HW group and all infants in the NW group were introduced to complementary feeding ($p = 0.064$). The median contribution of complementary feeding to the total energy intake did not differ between the HW group and the NW group (15.9 vs. 23.0%, $p = 0.37$).

Differences in HMO Composition Between HW and NW Groups

Human milk oligosaccharide (HMO) composition in Secretor and Non-secretor mothers differ significantly (17). Thus, HMO data were compared between the HW and NW; first stratified by secretor status and subsequently for Secretors and Non-secretors combined. Eight out of the 11 mothers with infants in the HW group and 15 of the 17 mothers with infants in the NW group were Secretors. As there were only five Non-secretors in total, we did not do separate analysis for these.

Differences in HMO content between the HW and NW groups at 5 and 9 months in milk from Secretor mothers alone are shown in **Table 2**. At 5 months, total HMO-bound fucose ($p = 0.033$) and DFLac ($P = 0.045$) were higher in the HW group compared to the NW group, whereas DFLNH ($p = 0.045$) and LNnT ($p = 0.012$) were lower in the HW group. No differences were observed for other HMOs. Total HMO-bound fucose remained significantly higher and LNnT concentrations remained significantly lower in the HW group at 9 months compared to the NW group, whereas no differences were observed for other HMOs. The most abundant HMO, 2′-FL was 36% higher in the HW group, but the difference was only borderline significant ($p = 0.061$). Overall, the results were similar between testing absolute concentrations and relative abundance of HMOs (data not shown).

At 5 months, when combining Secretors and Non-secretors, LNnT concentrations were lower in the HW group ($P = 0.046$), similarly as for Secretors only (**Supplemental Table 1**). Additionally, LSTc concentrations ($P = 0.041$) and HMO diversity ($P = 0.041$) were lower in the HW group compared to the NW group. No differences were observed at 9 months.

When also considering the 24 h milk volume, there were no differences between the groups for absolute intake of HMOs (mg/d) at 5 months.

Associations Between HMO Composition and Growth

Associations between HMO composition and anthropometry at 5 months and weight velocities from birth to 5 months

TABLE 3 | Spearman correlations between human milk oligosaccharide content and Height-for-Age Z-scores (HAZ) and BMI-for-Age Z-scores (BAZ) at 5 months (Secretors only)[a].

HMOs	HAZ		BAZ	
	Rho	*p*-value	Rho	*p*-value
Fucosylated or sialylated lactose				
2′-FL	0.238	0.275	0.388	0.067
3-FL	0.221	0.311	0.209	0.338
DFLac	0.501	**0.015**	0.128	0.562
3′-SL	0.461	**0.027**	−0.014	0.948
6SL	−0.222	0.309	−0.438	**0.036**
Non-fucosylated, non-sialylated HMOs				
LNT	−0.217	0.319	−0.225	0.301
LNnT	−0.541	**0.008**	−0.330	0.125
LNH	0.047	0.83	−0.221	0.311
Fucosylated, non-sialylated HMOs				
LNFP I	−0.296	0.171	0.051	0.818
LNFP II	0.139	0.527	−0.046	0.833
LNFP III	−0.290	0.180	−0.248	0.255
DFLNT	0.218	0.317	−0.115	0.602
FLNH	−0.097	0.660	−0.340	0.112
DFLNH	−0.475	**0.022**	−0.403	0.057
Non-fucosylated, sialylated HMOs				
LSTb	−0.186	0.396	−0.040	0.856
LSTc	−0.334	0.119	−0.202	0.355
DSLNT	−0.176	0.422	0.040	0.856
DSLNH	0.006	0.977	−0.329	0.126
Fucosylated, sialylated HMOs				
FDSLNH	−0.020	0.927	−0.343	0.109
HMO-bound fucose	0.374	0.079	0.357	0.094
HMO-bound sialic acid	0.121	0.582	−0.135	0.539
HMO sum	0.230	0.290	0.379	0.074
Diversity	−0.240	0.271	−0.378	0.075

[a]*Data are Spearman's correlation coefficient Rho and p-values. mo, months; 2′-FL, 2′-fucosyllactose; 3-FL, 3-fucosyllactose; 3′-SL, 3′-sialyllactose; 6′-SL, 6′-sialyllactose; LNT, lacto-N-tetraose; LNnT, lacto-N-neotetraose; LNFP I-II-III, lacto-N-fucopentaose; LSTb, LSTc, sialyl-lacto-N-tetraose; DSLNT, disialyl-lacto-N-tetraose; DFLac, difucosyl-lactose; DFLNT, difucosyl-lacto-N-tetraose; LNH, lacto-N-hexaose; FLNH, fucosyl-lacto-N-hexaose; DFLNH, difucosyl-lacto-N-hexaose; FDSLNH, fucosyl-disialyl-lacto-N-hexaose; DSLNH, disialyl-lacto-N-hexaose; HMOs, human milk oligosaccharides. Bold values indicate significance at a P < 0.05 level.*

TABLE 4 | Spearman correlations between human milk oligosaccharide content and weight velocity from 0 to 5 months (grams per week) (Secretors only)[a].

HMOs	Weight velocity 0–5 mo (grams pr. week)	
	Rho	*p*-value
Fucosylated or sialylated lactose		
2′-FL	0.500	**0.015**
3-FL	0.330	0.124
DFLac	0.417	**0.048**
3′-SL	0.261	0.229
6′-SL	−0.302	0.161
Non-fucosylated, non-sialylated HMOs		
LNT	−0.375	0.078
LNnT	−0.531	**0.009**
LNH	−0.134	0.541
Fucosylated, non-sialylated HMOs		
LNFP I	−0.102	0.644
LNFP II	−0.016	0.943
LNFP III	−0.285	0.188
DFLNT	−0.044	0.840
FLNH	−0.268	0.217
DFLNH	−0.502	**0.015**
Non-fucosylated, sialylated HMOs		
LSTb	−0.197	0.368
LSTc	−0.183	0.404
DSLNT	−0.142	0.517
DSLNH	−0.181	0.409
Fucosylated, sialylated HMOs		
FDSLNH	−0.243	0.264
HMO-bound fucose	0.565	**0.005**
HMO-bound sialic acid	−0.042	0.847
HMO sum	0.496	**0.016**
Diversity	−0.468	**0.024**

[a]*Data are Spearman's correlation coefficient Rho and p-values. mo, months; 2′-FL, 2′-fucosyllactose; 3-FL, 3-fucosyllactose; 3′-SL, 3′-sialyllactose; 6′-SL, 6′-sialyllactose; LNT, lacto-N-tetraose; LNnT, lacto-N-neotetraose; LNFP I-II-III, lacto-N-fucopentaose; LSTb, LSTc, sialyl-lacto-N-tetraose; DSLNT, disialyl-lacto-N-tetraose; DFLac, difucosyl-lactose; DFLNT, difucosyl-lacto-N-tetraose; LNH, lacto-N-hexaose; FLNH, fucosyl-lacto-N-hexaose; DFLNH, difucosyl-lacto-N-hexaose; FDSLNH, fucosyl-disialyl-lacto-N-hexaose; DSLNH, disialyl-lacto-N-hexaose; HMOs, human milk oligosaccharides. Bold values indicate significance at a P < 0.05 level.*

TABLE 5 | Spearman correlations between human milk oligosaccharide content and fat mass index (FMI) and fat free mass index (FFMI) at 5 months (Secretors only)[a].

HMOs	FMI 5 mo		FFMI 5 mo	
	Rho	*p*-value	Rho	*p*-value
Fucosylated or sialylated lactose				
2′-FL	0.468	**0.024**	0.245	0.259
3-FL	0.278	0.200	0.008	0.970
DFLac	0.280	0.196	−0.071	0.749
3′-SL	0.117	0.596	−0.247	0.256
6′-SL	−0.373	0.080	−0.338	0.115
Non-fucosylated, non-sialylated HMOs				
LNT	−0.304	0.158	−0.061	0.783
LNnT	−0.447	**0.033**	−0.139	0.526
LNH	−0.241	0.268	−0.294	0.173
Fucosylated, non-sialylated HMOs				
LNFP I	0.002	0.993	0.261	0.228
LNFP II	−0.055	0.802	−0.158	0.472
LNFP III	−0.331	0.123	−0.212	0.333
DFLNT	−0.100	0.650	−0.132	0.547
FLNH	−0.406	0.054	−0.223	0.307
DFLNH	−0.434	**0.039**	−0.192	0.381
Non-fucosylated, sialylated HMOs				
LSTb	−0.116	0.599	0.040	0.858
LSTc	−0.250	0.250	−0.206	0.345
DSLNT	−0.056	0.799	0.114	0.605
DSLNH	−0.323	0.133	−0.398	0.060
Fucosylated, sialylated HMOs				
FDSLNH	−0.342	0.110	−0.424	**0.044**
HMO-bound fucose	0.489	**0.018**	0.155	0.479
HMO-bound sialic acid	−0.129	0.556	−0.275	0.204
HMO sum	0.466	**0.025**	0.228	0.295
Diversity	−0.444	**0.034**	−0.256	0.239

[a]*Data are Spearman's correlation coefficient Rho and p-values. mo, months; 2′-FL, 2′-fucosyllactose; 3-FL, 3-fucosyllactose; 3′-SL, 3′-sialyllactose; 6′-SL, 6′-sialyllactose; LNT, lacto-N-tetraose; LNnT, lacto-N-neotetraose; LNFP I-II-III, lacto-N-fucopentaose; LSTb, LSTc, sialyl-lacto-N-tetraose; DSLNT, disialyl-lacto-N-tetraose; DFLac, difucosyl-lactose; DFLNT, difucosyl-lacto-N-tetraose; LNH, lacto-N-hexaose; FLNH, fucosyl-lacto-N-hexaose; DFLNH, difucosyl-lacto-N-hexaose; FDSLNH, fucosyl-disialyl-lacto-N-hexaose; DSLNH, disialyl-lacto-N-hexaose; HMOs, human milk oligosaccharides. Bold values indicate significance at a P < 0.05 level.*

were analyzed by combining the HW and NW group and excluding Non-secretors (**Tables 3–5**). Additional analyses were done combining Secretors and Non-secretors (**Supplemental Tables 2–4**).

2′-FL, the most abundant HMO, was positively associated with 0–5 months weight velocity ($p = 0.015$) and with FMI at 5 months ($p = 0.024$) (**Figure 1**). A similar direction for the association with change in WAZ from birth to 5 months was seen, but it did not reach statistical significance (Rho = 0.355, $p = 0.09$). DFLac was also positively associated with weight velocity ($p = 0.048$) and with length at 5 months ($p = 0.015$). 3′-SL was positively associated with length at 5 months ($p = 0.027$). 6′SL was the only HMO in the group of fucosylated or sialylated lactose HMOs which was inversely associated with anthropometry. It was inversely associated with BAZ at 5 months ($p = 0.036$) and tended to be inversely associated with FMI at 5 months ($p = 0.08$).

In the group of non-fucosylated, non-sialylated HMOs, only LNnT was associated with anthropometry. There was an inverse association with length ($p = 0.008$), with weight velocity ($p = 0.009$) and FMI ($p = 0.033$) (**Figure 2**). Again a trend for an inverse association with change in WAZ from birth to 5 months was observed (Rho = −0.389, $p = 0.07$). When combining Secretors and Non-secretors, LNnT was still inversely associated with length ($p = 0.018$, **Supplemental Table 2**).

Total HMO-bound fucose was positively associated with weight velocity 0–5 months ($p = 0.005$) and FMI ($p = 0.018$)

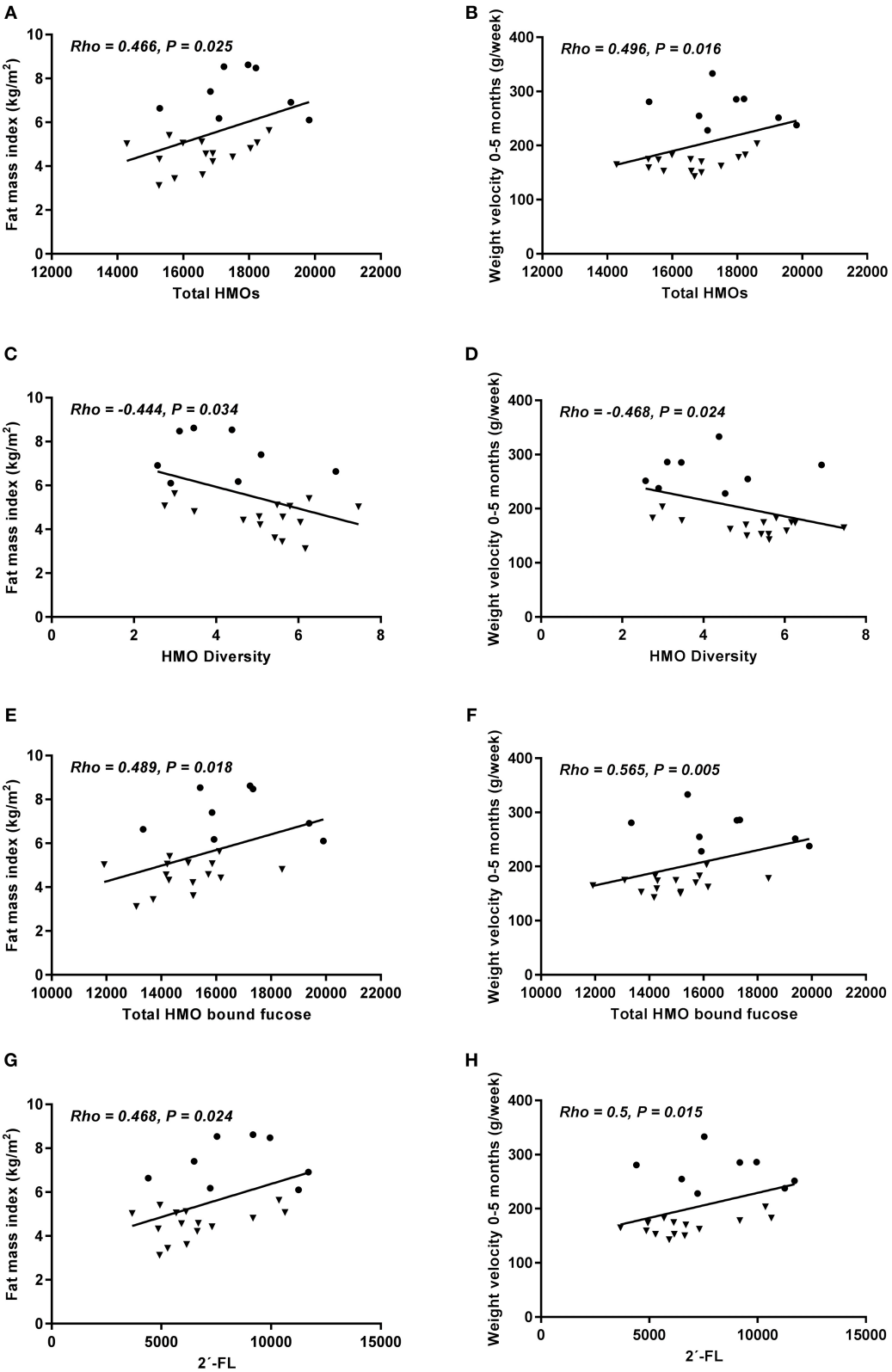

FIGURE 1 | Total HMO content **(A,B)**, HMO diversity **(C,D)**, HMO-bound fucose **(E,F)**, and 2′-FL concentration **(G,H)** at 5 months in relation to fat mass index at 5 months and weight velocity 0–5 months (grams pr. week). Only secretors included. Correlations were calculated using Spearman's rank correlation, but depicted as Pearson's correlation ($n = 23$). HW = • NW = ▼.

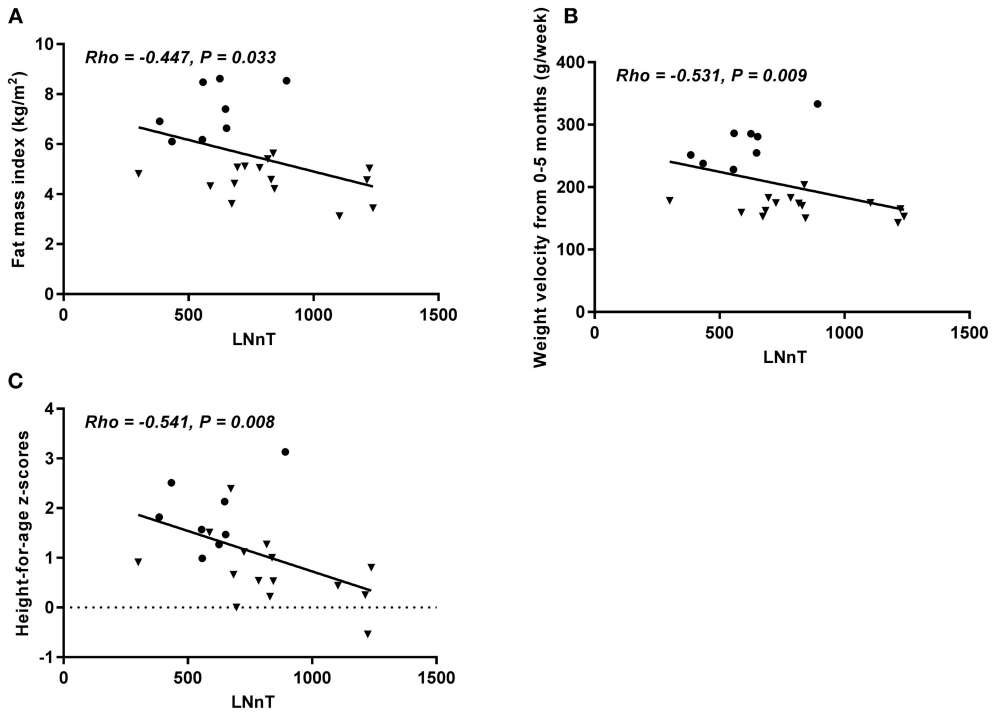

FIGURE 2 | Concentration of LNnt at 5 months in relation to fat mass index at 5 months **(A)**, weight velocity from 0 to 5 months (grams pr. week) **(B)**, and height-for-age Z-score **(C)**. Only Secretors included. Correlations were calculated using Spearman's rank correlation, but depicted as Pearson's correlation ($n = 23$). HW = • NW = ▼.

(**Figure 1**). Total HMO was positively associated with weight velocity 0–5 months ($p = 0.016$) and FMI at 5 months ($p = 0.025$), while the association between these two measures and HMO diversity were inverse ($p = 0.024$ and 0.034, respectively) (**Figure 1**). Both for total HMO-bound fucose and total HMO a similar direction for the association with change in WAZ from birth to 5 months was observed, but it did not reach statistical significance (Rho = 0.373, $p = 0.08$ and Rho = 0.338, $p = 0.10$, respectively).

When combining Secretors and Non-secretors, HMO diversity was still inversely associated with BAZ ($p = 0.046$), weight velocity ($p = 0.023$) and FMI at 5 months ($p = 0.024$) (**Supplementary Tables 2–4**).

Maternal Determinants of HMO Content

Maternal pre-pregnancy BMI, gestational weight gain, and BMI at 5 months were examined as potential determinants of HMO concentration in human milk.

In Secretor women, none of the HMOs were significantly associated with any of these three maternal determinants (**Table 6**).

When combining Secretors and Non-secretors, maternal BMI at 5 months was significantly negatively associated with 6′-SL ($P = 0.032$), and LSTb ($P = 0.03$), and positively associated with 2′-FL ($P = 0.017$), total HMO ($P = 0.015$), and total HMO-bound fucose ($P = 0.033$) (**Table 7**). Associations with pre-pregnancy BMI were in the same directions but tended to be weaker than the associations with maternal BMI at 5 months. We did not find any associations between any HMOs and weight gain in pregnancy (A6).

DISCUSSION

The aim of the study was to examine if differences in oligosaccharide composition in human milk were associated with the excessive weight gain observed in some exclusively breastfed infants, which had not been done before. We found only borderline significant differences between the HW and NW groups in total HMO concentration and HMO diversity, indicating higher total HMO and lower diversity in the HW group. However, concentrations of several individual HMOs were significantly different between the two groups suggesting that the variation in HMO composition could indeed be part of the explanation for the differences in growth between the two groups. This was further supported by the results showing that several HMOs were associated with anthropometry, growth velocity, and body composition in an analysis combining the HW and NW groups.

Our results indicate that certain HMOs might play a role in infant growth, which is in accordance with previously reported data. In a study by Alderete et al., LNFP I in mother's milk was negatively associated with infant weight at both 1 and 6 months of age, and with lean mass and fat mass (FM)

TABLE 6 | Maternal determinants of HMO content at 5 months (Secretors only)[a].

	Maternal BMI (5 mo)		Prepregnancy BMI		Weightgain in pregnancy	
HMOs	**Rho**	***p*-value**	**Rho**	***p*-value**	**Rho**	***p*-value**
Fucosylated or sialylated lactose						
2′-FL	0.336	0.117	0.279	0.198	−0.010	0.964
3-FL	0.098	0.657	0.039	0.861	0.027	0.903
DFLac	0.116	0.599	0.243	0.264	−0.326	0.129
3′-SL	−0.024	0.914	0.080	0.717	−0.149	0.498
6′-SL	−0.349	0.103	−0.271	0.211	−0.157	0.475
Non-fucosylated, non-sialylated HMOs						
LNT	−0.155	0.480	−0.049	0.823	0.035	0.873
LNnT	−0.123	0.578	−0.202	0.356	0.249	0.251
LNH	−0.190	0.386	−0.151	0.491	0.071	0.748
Fucosylated, non-sialylated HMOs						
LNFP I	0.100	0.650	−0.039	0.861	0.131	0.550
LNFP II	−0.167	0.446	−0.055	0.802	−0.132	0.549
LNFP III	−0.104	0.638	−0.139	0.526	0.216	0.322
DFLNT	−0.379	0.074	−0.225	0.301	−0.233	0.284
FLNH	−0.032	0.886	0.029	0.897	0.131	0.550
DFLNH	0.122	0.581	−0.072	0.744	0.226	0.301
Non-fucosylated, sialylated HMOs						
LSTb	−0.256	0.239	−0.268	0.217	0.055	0.805
LSTc	0.086	0.697	0.086	0.697	0.166	0.449
DSLNT	−0.163	0.457	−0.121	0.584	−0.001	0.996
DSLNH	−0.164	0.455	−0.003	0.989	0.062	0.777
Fucosylated, sialylated HMOs						
FDSLNH	−0.180	0.412	−0.155	0.480	0.072	0.744
HMO-bound fucose	0.285	0.188	0.273	0.208	−0.152	0.488
HMO-bound sialic acid	−0.299	0.165	−0.204	0.352	−0.021	0.923
HMO sum	0.374	0.079	0.308	0.152	−0.030	0.893
Diversity	−0.321	0.135	−0.266	0.220	−0.029	0.896

[a]*Data are Spearman's correlation coefficient Rho and p-values. mo, months; 2′-FL, 2′-fucosyllactose; 3-FL, 3-fucosyllactose; 3′-SL, 3′-sialyllactose; 6′-SL, 6′-sialyllactose; LNT, lacto-N-tetraose; LNnT, lacto-N-neotetraose; LNFP I-II-III, lacto-N-fucopentaose; LSTb, LSTc, sialyl-lacto-N-tetraose; DSLNT, disialyl-lacto-N-tetraose; DFLac, difucosyl-lactose; DFLNT, difucosyl-lacto-N-tetraose; LNH, lacto-N-hexaose; FLNH, fucosyl-lacto-N-hexaose; DFLNH, difucosyl-lacto-N-hexaose; FDSLNH, fucosyl-disialyl-lacto-N-hexaose; DSLNH, disialyl-lacto-N-hexaose; HMOs, human milk oligosaccharides.*

at 6 months of age. In contrast, DSLNT and LNFP II were positively associated with FM at 6 months, whereas, FDSLNH and LNnT were associated with higher and lower %FM at 6 months, respectively. Further, at 6 months DSLNT was negatively associated with body length (6). The group also reported that higher HMO diversity at 1 month was associated with lower %FM and FM at 1 month. In accordance with these findings, we found HMO diversity to be inversely associated with FMI at 5 months. These findings could indicate that HMO diversity influences infant body composition during exclusive breastfeeding. Furthermore, HMO diversity was also associated with weight velocity in our study and diversity was considerably lower in the HW group (3.92 vs. 5.48), but only borderline significant. However, Sprenger et al. found no differences in infant anthropometric measurements between infants consuming human milk with low or high fucosyltransferase 2 (FUT2, secretor) genotype-associated HMO concentrations or composition in a group of 50 mother-infants dyads (18). However, their study did not analyze HMOs that are independent of FUT2.

The only HMO that was linked to growth in both our study and the Alderete study was LNnT. In both studies it seems to be related to lower FM% as Alderete et al. (6) found negatively associations with FM% and we found lower values of LNnT in the HW group, which had a significantly higher %FM compared to the NW group. Furthermore, we found a negative association of LNnT with FMI at 5 months. LNnT has been shown to alter the gut microbiota in humans, which could explain why it is associated with growth (19).

The most plausible mechanism linking HMOs and growth is that HMOs play a role in developing the infant microbiome. Without being degraded by the infant digestive system, HMOs reach distal parts of the infant's intestine where they are metabolized by the intestinal microbiota (20). The gut microbiota might potentially play a role in energy harvest from the diet and in energy storage for the host and has been associated

TABLE 7 | Maternal determinants of HMO content at age 5 months (Secretors and Non-secretors combined)[a].

HMOs	Maternal BMI (5 mo)		Prepregnancy BMI		Weightgain in pregnancy	
	Rho	*p*-value	Rho	*p*-value	Rho	*p*-value
Fucosylated or sialylated lactose						
2′-FL	0.446	**0.017**	0.389	**0.041**	0.027	0.893
3-FL	0.299	0.122	0.235	0.229	0.069	0.726
DFLac	0.301	0.120	0.346	0.071	−0.176	0.371
3′-SL	0.172	0.382	0.218	0.264	−0.025	0.901
6′-SL	−0.406	**0.032**	−0.323	0.094	−0.131	0.507
Non-fucosylated, non-sialylated HMOs						
LNT	−0.327	0.089	−0.230	0.238	−0.059	0.765
LNnT	−0.177	0.368	−0.224	0.251	0.117	0.552
LNH	0.078	0.692	0.078	0.692	0.155	0.431
Fucosylated, non-sialylated HMOs						
LNFP I	0.299	0.122	0.195	0.319	0.130	0.51
LNFP II	−0.361	0.059	−0.278	0.152	−0.161	0.413
LNFP III	−0.198	0.314	−0.211	0.28	0.097	0.624
DFLNT	−0.055	0.782	0.061	0.759	−0.161	0.414
FLNH	0.158	0.421	0.198	0.314	0.319	0.098
DFLNH	0.019	0.923	−0.131	0.507	0.203	0.300
Non-fucosylated, sialylated HMOs						
LSTb	−0.411	**0.030**	−0.403	**0.034**	−0.031	0.875
LSTc	0.342	0.075	0.325	0.091	0.217	0.267
DSLNT	−0.182	0.355	−0.152	0.440	−0.022	0.911
DSLNH	−0.203	0.300	−0.063	0.748	0.026	0.896
Fucosylated, sialylated HMOs						
FDSLNH	−0.257	0.187	−0.242	0.215	0.108	0.583
HMO-bound fucose	0.404	**0.033**	0.373	0.051	−0.054	0.787
HMO-bound sialic acid	−0.276	0.155	−0.215	0.272	0.032	0.871
HMO sum	0.455	**0.015**	0.393	**0.039**	0.019	0.925
Diversity	−0.257	0.187	−0.231	0.237	0.032	0.870

[a]*Data are Spearman's correlation coefficient Rho and p-values. mo, months; 2′-FL, 2′-fucosyllactose; 3-FL, 3-fucosyllactose; 3′-SL, 3′-sialyllactose; 6′-SL, 6′-sialyllactose; LNT, lacto-N-tetraose; LNnT, lacto-N-neotetraose; LNFP I-II-III, lacto-N-fucopentaose; LSTb, LSTc, sialyl-lacto-N-tetraose; DSLNT, disialyl-lacto-N-tetraose; DFLac, difucosyl-lactose; DFLNT, difucosyl-lacto-N-tetraose; LNH, lacto-N-hexaose; FLNH, fucosyl-lacto-N-hexaose; DFLNH, difucosyl-lacto-N-hexaose; FDSLNH, fucosyl-disialyl-lacto-N-hexaose; DSLNH, disialyl-lacto-N-hexaose; HMOs, human milk oligosaccharides. Bold values indicate significance at a P < 0.05 level.*

with overweight and obesity. Studies have shown that a specific microbiome pattern can have an increased ability to harvest energy from the diet and thus accelerate growth (5, 21, 22). Other mechanisms linking HMOs and growth have also been suggested such as HMOs having direct effects on epithelial cell responses in the gut or that HMOs have systemic effects, by being absorbed (partly) intact into circulation (6).

Several studies have shown a distinctively different fecal microbiota composition in breastfed compared with formula-fed infants (23, 24). Furthermore, the composition of the fecal microbiota in breastfed infants were correlated with the HMO composition in the milk consumed in other studies (25).

There is increasing interest in adding HMOs to infant formula in order to optimize the intestinal microbiota and the development of the immune system. Since HMOs might influence energy harvest through alterations of the microbiota, HMOs might also affect growth. Therefore, there has been an interest in testing if the addition of HMOs to infant formula has an effect on growth. In a multicenter randomized trial, growth was compared between two groups of infants (age < 14 days) randomized to receive either a formula supplemented with LNnT and 2′-FL or a standard formula, from enrolment to 6 months of age (7). Weight gain was the same in the two groups and they concluded that a formula with these two HMOs added supports age-appropriate growth. Interestingly, we found that in the human milk the HW group received, LNnT was significantly lower both at 5 and 9 months, and was strongly negatively associated with weight velocity from 0 to 5 months and with length at 5 months (both $p < 0.009$) in the combined group. Furthermore, the content of the most abundant HMO, 2′-FL was 36% higher in the HW group compared to the NW group, though only borderline significant ($p = 0.061$), and positively associated with weight gain from 0 to 5 months and with FMI at 5 months. Thus, the effect on growth of 2′-FL and LNnT seems to be opposite, which might be the reason that adding both of these to a formula did not influence growth (7). However, another study adding 2′-FL to infant formula showed that infants in the intervention study had a growth pattern not different from breastfed infants and a review also concluded that adding

2′-FL or LNnT seems to support normal growth, although the data are limited (26, 27). In the two studies adding 2′-FL to formula the amount was 1.0 g/L (7, 27), which is considerable lower than the average 2′-FL concentration in our NW (∼3 g/L) and HW group (∼4 g/L) (**Table 2**). In the study adding two HMOs to infant formula (7) the amount of LNnT was 0.5 g/L, which is about the same concentrations as in human milk in our NW group.

The potential maternal determinants of HMO concentrations were examined in an analysis combining the HW and NW groups. Pre-pregnancy BMI and maternal BMI at the time of HMO sampling were associated with some HMOs whereas gestational weight gain was not. These associations were found in the analysis including both Secretors and Non-secretors whereas no associations were found in the analysis including only Secretors. We did not analyze Non-secretors separately since we only had very few infants in this group. Maternal weight and body composition have recently been linked to growth in infancy in 427 mother-infant dyads, showing that LNH and DFLNT concentrations were higher in overweight and obese mothers compared with normal weight mothers (17). In our study, where only few of the mothers were overweight or obese (11), we found that maternal BMI at 5 months was positively associated with total HMO, HMO-bound fucose as well as the most abundant HMO 2′-FL and negatively associated with LSTb and 6′-SL.

Our study has some limitations with the main limitation being the small sample size, which limits the power to detect differences between groups. Furthermore, we did not adjust for multiple testing as this is an exploratory study and our findings therefore, need to be verified in other studies. In addition, the study was a single center study further limiting the generalizability to other settings. Another limitation is that it was not possible to identify, recruit, and arrange clinical visits before the age of 5 to 6 months when growth velocity was decreasing and some infants had been introduced to complementary foods. HMO composition is likely to change during the course of lactation, as shown by Alderete et al. where 13 out of 16 analyzed HMOs changed significantly from 1 to 6 months post-partum (6) as well as in a cross-sectional study by Azad et al. (17) that revealed associations between HMO concentrations and days of lactation. Therefore, the HMO composition measured at 5–6 months might not fully reflect the HMO composition during the entire period where the excessive weight gain took place (0–5 months). If we had been able to measure HMOs when growth velocity was considerably higher, it is possible that we had found stronger associations between growth and HMOs, but this is speculative. Adjusting for other milk components such as fat, lactose, and protein content was not possible in the present study due to the small sample size, however, in an earlier study, minimal differences between the two groups were found and thus we think that this is unlikely to have affected the current results.

An additional limitation of the current study is the lack of long term follow up that would add additional information on the long-term effects of the excessive weight gain in early life as well as associations between HMOs and long-term growth patterns of children. Finally, long-term follow ups are essential for understanding the long-term consequences of excessive weight gain observed in some exclusively breastfed infants.

The strengths of the study are that we use a state of the art method for assessing HMOs in a well-characterized cohort. Although the number of participants was low, the fact that we have included infants with excessive weight gain and compared them with a group with a normal weight gain increases the chances of finding associations between HMO intake and growth. Furthermore, we have estimated data on total milk intake, which is the first study that not only looks at HMO concentrations but also computes total HMO intake considering both concentration and volume. However, it should be emphasized that milk intake was measured after the excessive weight gain occurred and is therefore subject to the same limitations as the HMO content discussed above. Future studies should be performed in larger samples with longer follow-up to identify the contribution of specific HMOs to infant growth and development. Furthermore, the link between HMOs and gut microbiota in relation to growth should be investigated simultaneously and in both human studies and experimental models to confirm a causal link. Moreover, determinants of HMO concentrations should be investigated to find potential modifiable factors, which could be targeted in interventions.

CONCLUSIONS

In conclusion, we found significant differences between HMO concentrations in a group of exclusively breastfed infants with high weight gain compared to a group of infants with normal weight gain, which emphasizes that HMOs play an important role in infant growth. However, since this is a small explorative study it cannot proof causality, warranting further in-depth studies that investigate the role and underlying mechanisms that link variation in HMO composition to excessive weight gain. Understanding the link between HMOs and infant body composition, growth, and potential long-term consequences will be of paramount importance before individual HMOs are added to formula fed to infants.

DATA AVAILABILITY

The datasets for this study will not be made publicly available because in this small cohort through the growth pattern of the individual infants it will be possible to identify the infants.

ETHICS STATEMENT

The study protocol was approved by the Regional Ethical Committee of the Capital Region of Denmark in accordance with the Helsinki declaration (H-15008948) and the Data Protection Agency (2015-57-0117 and 2015-57-0116) and written informed consent was obtained from all parents.

AUTHOR CONTRIBUTIONS

MWL, AL, CM, KM, and LB: conceptualization. MWL, MVL, and RL: formal analysis. CM, KM, and LB: funding acquisition. MWL: investigation and project administration. MWL, AL, CM, KM, LB, and CY: methodology. MWL, MVL, KM, and LB: writing—original draft. MWL, MVL, RL, CY, AL, CM, KM, and LB: writing—review and editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2019.00297/full#supplementary-material

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CLINICAL TRIAL
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Feeding Infants Formula With Probiotics or Milk Fat Globule Membrane: A Double-Blind, Randomized Controlled Trial

*Xiaonan Li[1], Yongmei Peng[2], Zailing Li[3], Britt Christensen[4], Anne B. Heckmann[5], Hans Stenlund[6], Bo Lönnerdal[7] and Olle Hernell[8]**

[1] Department of Children Health Care, Children's Hospital of Nanjing Medical University, Nanjing, China, [2] Department of Children Health Care, Children's Hospital of Fudan University, Shanghai, China, [3] Department of Pediatrics, Peking University Third Hospital, Beijing, China, [4] Arla Foods amba, Arla Innovation Center, Skejby, Denmark, [5] Arla Foods Ingredients Group P/S, Viby J, Denmark, [6] Epidemiology and Global Health, Department of Public Health and Clinical Medicine, Umeå University, Umeå, Sweden, [7] Department of Nutrition, University of California, Davis, Davis, CA, United States, [8] Department of Clinical Sciences, Pediatrics, Umeå University, Umeå, Sweden

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Edited by:
Alexandra Papadopoulou, First Pediatric Clinic of the University of Athens, Children's Hospital Hagia Sophia, Greece

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Matthew Wyatt Carroll, University of Alberta, Canada

***Correspondence:**
Olle Hernell
olle.hernell@umu.se

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Purpose: To evaluate effects on growth and infection rates of supplementing infant formula with the probiotic *Lactobacillus paracasei* ssp. *paracasei* strain F19 (F19) or bovine milk fat globule membrane (MFGM).

Methods: In a double-blind, randomized controlled trial, 600 infants were randomized to a formula supplemented with F19 or MFGM, or to standard formula (SF). A breastfed group was recruited as reference ($n = 200$).The intervention lasted from age 21 ± 7 days until 4 months, and infants were followed until age one year.

Results: Both experimental formulas were well tolerated and resulted in high compliance. The few reported adverse events were not likely related to formula, with the highest rates in the SF group, significantly higher than for the F19-supplemented infants ($p = 0.046$). Weight or length gain did not differ during or after the intervention among the formula-fed groups, with satisfactory growth. During the intervention, overall, the experimental formula groups did not have more episodes of diarrhea, fever, or days with fever than the breastfed infants. However, compared to the breastfed infants, the SF group had more fever episodes ($p = 0.021$) and days with fever ($p = 0.036$), but not diarrhea. Compared with the breastfed group, the F19-supplemented infants but not the other two formula groups had more visits/unscheduled hospitalizations ($p = 0.015$) and borderline more episodes of upper respiratory tract infections ($p = 0.048$).

Conclusions: Both the MFGM- and F19-supplemented formulas were safe and well-tolerated, leading to few adverse effects, similar to the breastfed group and unlike the SF group. During the intervention, the MFGM-supplemented infants did not differ from the breastfed infants in any primary outcome.

Keywords: infant, breastfed, MFGM, F19, infection, safety, probiotics

INTRODUCTION

Breastfeeding is considered the "gold standard" for infant nutrition because human milk offers an adequate supply of nutrients and biologically active components with benefits for growth, development, and protection against infections (1). Infants fed standard formula (SF) are at higher risk of otitis media (2) and gastrointestinal and respiratory infections (3, 4). For this reason, a goal of infant formula development is to emulate the composition and functionality of breast milk to close this gap in health outcomes (5). Anti-infectious factors in human milk include immunoglobulins, anti-bacterial and anti-viral proteins, leukocytes, and oligosaccharides, which collectively are considered to reduce the risk of gastrointestinal and other infections in breastfed infants. Studies also strongly suggest that the gut microbiota is associated with positive health outcomes (6, 7). Diet is among the main drivers of the composition and function of the gut microbiota (8). In breastfed infants, bifidobacteria and lactobacilli dominate this microbiota, whereas formula-fed infants have a more diverse colonization, including *Bacteroidetes*, bifidobacteria, staphylococci, *Escherichia coli*, and *Clostridia* (9–12). Several meta-analyses have reported that supplementation with a probiotic may be beneficial in preventing and treating upper respiratory tract infections (13), infectious diarrhea, and antibiotic-induced diarrhea (14), as well as allergic disease, e.g., eczema in children (15). Some studies, however, have found no effect of probiotics (16–18). It seems reasonable to develop infant formulas that support establishment of a microbiota resembling that of breastfed infants through the addition of bioactive components or probiotics. A previous study indicated that supplementing with the *Lactobacillus paracasei* ssp. *paracasei* strain F19 (F19) during weaning could be an effective tool in prevention of early manifestations of allergy, such as eczema, in infants ages 4–13 months (19). Results of another study suggested a reduced risk of lower respiratory tract infections when this probiotic was combined with prebiotics (20). Collectively, these studies support that F19 is safe, even from the first months of life.

The milk fat globule membrane (MFGM) envelops the triglyceride-rich core of the milk fat globule when secreted from epithelial cells of the lactating mammary gland. This membrane contains numerous biologically active components (21, 22), many with antimicrobial effects, e.g., gangliosides (23), oligosaccharides (24), and the glycoproteins butyrophilin, lactadherin, and mucin (25, 26). By tradition, infant formulas have been produced from skim milk powder and whey protein concentrate, and the milk fat has been discarded. The fat is typically replaced by a blend of vegetable oils. For this reason, compared to breast milk, infant formulas contain much less of the biologically important MFGM proteins and lipids. Results of a growing number of clinical trials of MFGM supplementation for infants or children support positive effects on both neurodevelopment (27, 28) and defense against infections (29, 30). Bovine milk fractions enriched in MFGM are now commercially available, and infant formulas with MFGM have been launched in several countries.

The aim of the present study was to evaluate the effects of feeding infants a SF supplemented with either F19 or MFGM compared to feeding them unsupplemented SF, and using a breastfed group as reference with regard to infant growth and health. The primary hypothesis was that consumption of formula containing either F19 or MFGM would reduce the incidence of infections. Furthermore, we hypothesized that feeding infant formula with F19 or MFGM from the first months of life would be safe and tolerable.

Abbreviations: MFGM, milk fat globule membrane; F19, probiotic *L. paracasei* ssp. paracasei strain F19; SF, standard formula; AE, adverse event; SAE, serious adverse event.

METHODS

The study was conducted at several centers in China in Nanjing (Children's Hospital of Nanjing Medical University, Nanjing Maternity and Child Health Care Hospital, the Second Affiliated Hospital of Nanjing Medical University, Nanjing Secondary Hospital, and Huaian Maternity and Child Health Hospital), Shanghai (Children's Hospital of Fudan University, Clinical Center for Public Health of Fudan University), and Beijing (Peking University Third Hospital, Beijing Ditan Hospital Capital Medical University, and The First Hospital of Jilin University). It was approved by the institutional review board at the University of California, Davis, as well as the regional ethical review boards in Nanjing, Shanghai, and Beijing, China, and conducted according to the principles in the Declaration of Helsinki. Complete oral and written information about the study was given to the parents/caregivers, and written consent was obtained from the parents or caregivers of all infants before inclusion. The clinical trial was registered at ClinicalTrials.gov (NCT01755481).

Inclusion Criteria and Background Information

The study was a randomized, double-blind, controlled trial comparing three different infant formulas, with breastfed infants as the reference group. Statistical power calculations revealed that a sample size of 540 infants (180 in each group) was needed to detect a difference of 20% in incidence of infectious episodes, the primary outcome, with 80% power (5% significance). Anticipating a drop-out rate of 15–20%, our aim was to include 800 infants, 200 in each formula group and 200 breastfed infants. Infants were recruited consecutively from December 2013 to August 2016. Inclusion criteria for all infants were gestational age of 37–42 weeks at birth, birth weight >2,500 g and <4,000 g, absence of chronic illness, and a parent or legal representative who could speak and understand Chinese. Exclusion criteria for all infants were malformations, handicaps, or congenital diseases that could affect normal feeding or growth, treatment with antibiotics (including perinatal treatment of the mother), and having been fed infant formula with pre- and/or probiotics. Inclusion criteria for the formula-fed group were healthy infants of mothers who could not or voluntarily completely refrained from breastfeeding at inclusion (infant age 21 ± 7 days).

The exclusion criterion for the formula-fed groups was any breastfeeding at the age of 28 days. Inclusion criterion for the breastfed group was having been exclusively breastfed from birth and mothers intending to breastfeed >80% to age at least 4 months. Exclusion criteria for the breastfed group were infants fed >20% infant formula of their calculated total intake at 28 days of age. Background information was collected at the time of recruitment. Information on birth weight, feeding pattern, and parental education was recorded for all excluded and drop-out infants.

Composition of Infant Formulas

Formulas were manufactured from bovine milk powder by Arla Foods amba, Denmark. The probiotic bacterium *L. paracasei*, ssp. *paracasei* strain F19 was from Chr. Hansen, Denmark, and Lacprodan® MFGM-10 from Arla Foods Ingredients group P/S, Denmark. The composition of each of the three formulas is shown in **Table 1**. The final study formulas were produced in Hohhot, China, in accordance with Chinese regulations under strict hygienic conditions, adhering to all prerequisites for human consumption.

Randomization and Intervention

The intervention was blinded both to parents and staff until analyses were completed. Infants were randomized to one of the three infant formulas: SF; the same formula supplemented with F19 at a dose of $1*10^8$ cfu/L; or Lacprodan® MFGM-10 (3.88 g Lacprodan® MFGM-10/100 g powder, or 5 g/L prepared formula) from inclusion at 21 ± 7 days to the end of the fourth month. For randomization, a computerized randomization tool in blocks of 24 was used, stratifying for sex (12 boys and 12 girls) and type of formula coded by color (eight of each color). The block size for the breastfed group was eight (4 boys and 4 girls) in each group. Powdered formula was distributed to families together with preparation instructions in identical boxes marked with a color coded number, prepared at the manufacturing site before being sent to the study site. Prior to the start of intervention, infants were fed SF if formula feeding had been started.

From the beginning of the fifth month to the end of the sixth month of age, all infants in the formula groups received SF. If breast milk supply was insufficient, breastfed infants were fed SF, but not exceeding 20% of their calculated total intake based on the 3-day formula intake record (see below). Complementary foods were not allowed during the intervention but were introduced no later than 26 weeks of age, according to current recommendations. Vitamin D supplements were given according to current recommendations.

Assessment of Growth

Visits were made at baseline (inclusion) and at 1, 2, 3, 4, 5, 6, 9, and 12 months of age. At each visit, weight, length, and head circumference were measured. Weight was assessed to the nearest 10 g. The same electronic weighing scales (Seca 757; Seca, Germany) were used for all infants at all visits at each center and calibrated at the first visit and every visit thereafter until the end of the study. Recumbent length was measured to nearest 1 mm using a standardized length board (Seca 416; Seca, Germany). Head circumference was measured to the nearest 1 mm using a standard non-elastic plastic-coated measuring tape (Seca 212,

TABLE 1 | Composition of infant formulas used in the study.

	SF[a]	MFGM	F19
Energy (kcal/100 mL)	66	67	66
Protein (g/100 mL)	1.6	1.5	1.6
Casein (g/100 mL)	0.60	0.59	0.60
Whey (g/100 mL)	0.99	0.95	0.97
Carbohydrate (g/100 mL)	7.0	7.3	7.0
Fat (g/100 mL)	3.5	3.6	3.5
Linoleic acid (g/100 mL)	0.7	0.7	0.7
α-Linolenic acid (mg/100 mL)	64	65	64
DHA[b] (% of total fatty acids)	0.33	0.31	0.29
ARA (% of total fatty acids)	0.45	0.43	0.39
Minerals			
Sodium (mg/100 mL)	20	20	20
Potassium (mg/100 mL)	67	62	67
Copper (μg/100 mL)	61	59	61
Magnesium (mg/100 mL)	8.1	8.0	8.1
Iron (mg/100 mL)	0.81	0.81	0.81
Zinc (mg/100 mL)	0.6	0.6	0.6
Manganese (μg/100 mL)	8.5	8.1	8.5
Calcium (mg/100 mL)	50	49	50
Phosphorus (mg/100 mL)	36	34	36
Iodine (μg/100 mL)	11	12	11
Chloride (mg/100 mL)	47	48	47
Selenium (μg/100 mL)	2.5	2.3	2.5
Vitamins			
Vitamin C (mg/100 mL)	9.1	9.3	9.1
Vitamin A (μgRE/100 mL)	85	81	85
Vitamin E (mg α-TE/100 mL)	1.2	1.2	1.2
Vitamin D (μg/100 mL)	1.0	1.1	1.0
Vitamin K1 (μg/100 mL)	5.4	5.2	5.4
Vitamin B1 (μg/100 mL)	86.1	88.0	86.1
Vitamin B2 (μg/100 mL)	211	159	211
Vitamin B6 (μg/100 mL)	80.3	72.4	80.3
Vitamin B12 (μg/100 mL)	0.4	0.3	0.4
Niacin (μg/100 mL)	742	761	742
Folic acid (μg/100 mL)	17.0	15.7	17.0
Pantothenic acid (μg/100 mL)	644	575	644
Biotin (μg/100 mL)	2.8	2.4	2.8
Optional ingredients			
Choline (mg/100 mL)	10.7	8.9	10.7
Inositol (mg/100 mL)	4.6	4.8	4.6
Lutein (μg/100 mL)	9.6	8.8	9.6
Nucleotide[c] (mg/100 mL)	2.9	3.0	2.9
Taurine (mg/100 mL)	6.2	5.8	6.2
L-carnitine (mg/100 mL)	1.9	1.7	1.9

[a] *SF, standard formula; MFGM, formula supplemented with Lacprodan® MFGM-10; F19, formula supplemented with L. paracasei ssp. paracasei strain F19.*
[b] *DHA, docosahexaenoic acid; ARA, arachidonic acid.*
[c] *A mixture of disodium salts of 5'-AMP, 5'-CMP, 5'-GMP, 5'-UMP, and 5'-IMP.*

Seca, Germany). Anthropometric data are presented as z-scores calculated from the World Health Organization reference growth standards for breastfed infants (31).

Formula Intake

Parents of formula-fed infants were asked to complete a 3-day formula intake diary every month from inclusion until the end of the fifth month of age. Parents or caregivers of breastfed infants were asked to note all formula fed to the infant to allow the study staff to check adherence with breast milk consumption.

Assessment of Episodes of Infections and Health

From inclusion until 12 months of age, the incidence and duration of infectious episodes (acute diarrhea, upper and lower acute respiratory tract infections, fever) were diagnosed and recorded by the study physician based on the following definitions: Acute diarrhea was defined as three or more watery stools within a 24-h period or loose-to-watery bowel movements that exceeded the infant's usual daily stool frequency by two or more stools. Acute respiratory infections were defined as presence of two or more of the following symptoms as reported by the parent/caretaker: nasal discharge (clear, cloudy, yellow, or green), cough, fever, rapid, labored and/or noisy breathing, wheezing, chest in-drawing, flaring of nostrils, ear pain and/or discharge, and cyanosis. Respiratory symptoms that occurred within 2 weeks of the beginning of the illness were defined as part of the same episode. Symptoms presented more than 2 weeks after the start of an incident were considered as a new episode. Parent-reported number of episodes and days with fever (>38°C), vomiting, use of antibiotics, unscheduled doctor's visits, and hospitalization (incidence, duration, diagnosis, and treatment) were registered based on reviews performed every second week by the study physician. The length of the period of antibiotic use was recorded. Stool consistency was registered as watery diarrhea, loose, soft formed, or hard in the monthly 3-day dietary and health record. Parents/caretakers whose infants dropped out were asked to remain in the study for follow-up on an intention-to-treat basis.

Definition of Adverse Event and Serious Adverse Event

An adverse event (AE) was defined as any untoward occurrence in an infant administered a test product and that did not necessarily have to have a causal relationship with the product. AEs were illnesses, signs, or symptoms (including an abnormal laboratory finding) occurring or worsening during the course of the study. A serious adverse event (SAE) was a fatal or life-threatening event causing permanent harm or requiring/extending inpatient treatment at a hospital, or that the physician considered medically relevant.

All AEs were documented on the case report form. In the case of a SAE persisting beyond the trial termination, a follow-up visit was required. Furthermore, study physicians analyzed each report for a potential cause–effect relationship between the study products and the AE. Cow's milk protein allergy was diagnosed by a physician as follows: elimination of cow's milk protein/formula with disappearance of symptoms and reappearance of the same symptoms on reintroduction of milk protein/formula. When diagnosed, infants were recommended a protein hydrolysate formula and were considered study drop-outs.

Blood Sample Collection and Storage

All infants had a venous blood sample of 0.5–2 mL collected by the study staff on two occasions, one at the end of the intervention when the infants were age 4 months. After centrifugation at 1,500 rpm for 10 min, serum was collected and immediately frozen and stored at −80°C until shipped on dry ice to the sites of analyses.

Stool Sample Collection and Storage

Parents/legal representatives were asked to collect stool samples at different time points, including at the end of the intervention period. Before collection of the first stool sample, a reusable isolated bag for transportation of the stool samples, a reusable freezing body, two containers for the stool samples, a plastic bag for the filled containers, gloves, and instructions for collection, storage, and transportation of the stool samples were provided to the parent or the infant's legal representative. Stool samples were collected in the containers, put in the plastic bag, and stored in a freezer (−20°C) until the day of the visit. The frozen stool samples were transported to the study site, where they were stored at −20°C until shipped on dry ice for analysis (TNO, The Hague, Netherlands).

Serum Ferritin

Serum ferritin was analyzed in infants at age 4 months, with an enzyme-linked immunosorbent assay kit (RayBiotech, Norcross, GA, USA). This kit uses a biotinylated antibody specific for human ferritin and horseradish peroxidase–conjugated streptavidin. Samples were run in duplicate, and values are presented as means.

Fecal DNA Extraction and qPCR

DNA from fecal samples collected at the end of the intervention was isolated as previously described (32) with some minor modifications. The samples were initially mixed with 250 μL lysis buffer (Agowa, Berlin, Germany), 250 μL zirconium beads (0.1 mm), and 200 μL phenol, before being introduced to a BeadBeater (BioSpec Products, Bartlesville, OK, USA) for two × 2 min. Quantitative PCR detection was performed with the primers and according to conditions described previously (33). To evaluate if samples contained F19, data were plotted from low to high Ct, resulting in an S-shaped curve, with true positives in the lower and true negatives in the higher end, as previously described (33).

Statistics

Comparison of means among the MFGM, and F19 groups and the SF group were done pair-wise with independent samples *t*-tests. In the case of skewed distributions, comparisons were performed with Mann–Whitney *U*-test. Categorical variables were compared pair-wise using the Chi square test or Fisher's exact test. Comparisons of treatment groups with the breastfed

group were also done pair-wise. Analysis of variance was used for analyzing ferritin concentrations among groups.

Only adjusted *p*-values are presented in the text. Adjustment was based on the Bonferroni method. Per-protocol analyses were based on the 674 children who completed the study. There were no significant differences among the three sites with respect to birth weight, sex distribution, or education level, or with respect to major outcomes. All calculations were done using SPSS v 23 (IBM SPSS Statistics, Armonk, NY). Significance level was set to 5%.

RESULTS

In total, 799 children were recruited and randomized to formula-fed groups or recruited to the breastfed reference group. Ten infants did not attend any of the visits. Thus, 789 children

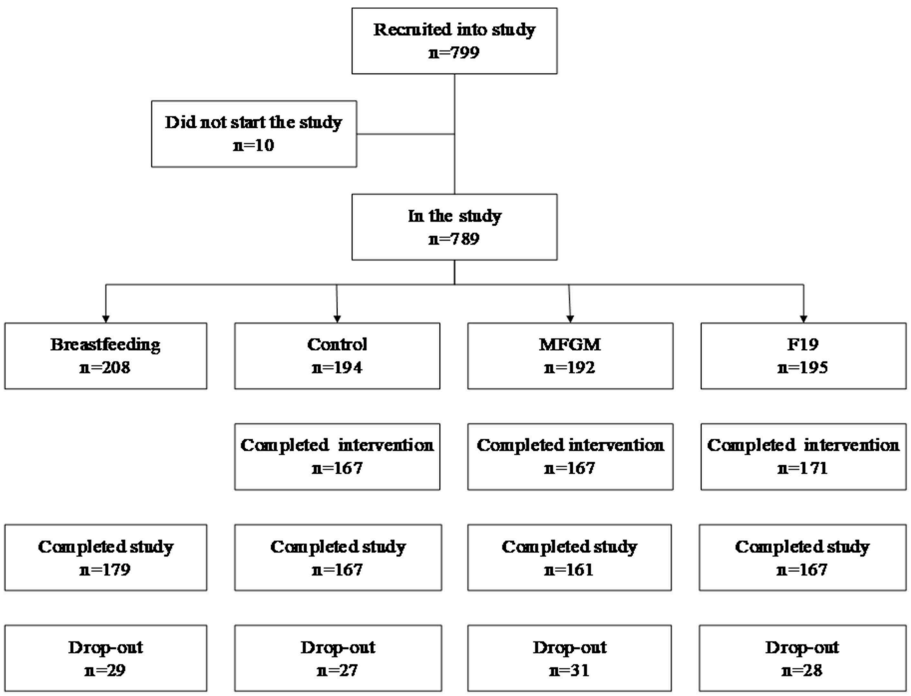

FIGURE 1 | Drop-out rates and adherence to the intervention in the formula-fed groups (SF, standard formula; MFGM, formula supplemented with milk fat globule membrane; F19, formula supplemented with *L. paracasei* ssp. *paracasei* strain F19) and the BF (breastfed) group. Ten children did not show up at any of the examinations and were excluded. During the study, 115 children left the study at various times, giving a total drop-out rate of 14.6% (BF, 14.1; SF, 14.1; MFGM, 17.9, and F19, 15.1%) with no significant difference among groups. The most common reason for drop-out (78%) was parent/caregiver decision to do so without explanation.

TABLE 2 | Demographic characteristics of the formula-fed and breastfed groups.

Variables	BF ($n = 208$)	SF ($n = 194$)	MFGM ($n = 192$)	F19 ($n = 195$)
Birth weight (g), mean (SD)	3381 (314)	3298 (374)	3279 (399)	3281 (412)
Sex, girls (%)	52.9	49.5	51.6	49.7
Gestational age (weeks)	39.0	38.7	38.8	38.8
Siblings (% with no siblings)	75.5	61.9	64.1	61.5
Delivery (% cesarean)	45.9	58.8	62.8	57.5
Pregnancy complications (%)	7.7	9.8	9.4	7.2
Mother's age (years), mean (SD)	29.4 (3.5)	29.6 (4.6)	29.2 (4.3)	29.5 (4.5)
Father's age (years), mean (SD)	31.3 (4.4)	31.4 (5.2)	31.1 (4.9)	31.6 (6.1)
Mother's education (%)	4.3/25.0/70.7	9.8/47.9/41.2	9.9/44.3/44.8	9.2/40.5/46.7
Father's education (%)	3.8/21.2/74.5	9.3/47.4/42.3	7.3/41.7/49.0	9.7/43.6/44.6

BF, breastfed reference group; SF, standard formula; MFGM, formula supplemented with milk fat globule membrane; F19, formula supplemented with L. paracasei ssp. paracasei strain F19; SD, standard deviation.

Education (%) refers to low/middle/high education level, i.e., ≤12 y/13–15y/ ≥ 16 y of school and university education.

remained for intention-to-treat analyses. In these analyses, missing continuous values were replaced according to "carry forward last value." Missing categorical values were replaced with zero (**Figure 1**).

Adherence

During the intervention, no formula consumption was reported for 22 formula-fed children, 42 children had consumption reported for 1, 2, or 3 months, and 505 children had consumption reported for the whole intervention. The proportion of children adherent for the whole intervention period did not differ significantly among the formula-fed groups. Average formula consumption during the intervention for the SF, MFGM, and F19 groups was 876, 866, and 833 mL/day, respectively. The F19 group consumed a lower average volume than the SF group ($p = 0.020$), but there was no difference between the SF and MFGM groups. Analysis of F19 in the stool in a randomized subsample of 100 from each of the formula-fed groups at age 4 months showed that 92% of the infants in the F19 group carried F19 compared to none in the other formula groups, confirming high adherence (data not shown).

Demographic Characteristics

Basic characteristics for the groups are shown in **Table 2**. There was no significant difference in birth weight, sex distribution, gestational age, type of delivery, pregnancy complications, parental education level, or proportion of no siblings among the formula-fed groups. However, birth weight ($p = 0.002$), parental education level ($p < 0.013$), and proportion of no siblings ($p = 0.001$) were lower for the formula-fed groups combined than for the breastfed group.

Anthropometrics

Weight z-scores did not differ significantly among the formula-fed groups at any time point. Mean weight for the breastfed group was significantly higher than for the F19 group until age 2 months ($p = 0.015$ and 0.028 at 1 and 2 months, respectively) and for the SF and MFGM groups until age 4 months (all $p \leq 0.041$). After these ages, the groups showed no significant differences (**Figure 2A** and **Table 3**). During the intervention, weight gain (g/day) did not differ among the formula-fed groups or between the formula-fed groups overall and the breastfed group. However, at 5–12 months, weight gain in the MFGM group was slightly (1.1 g/day) but significantly higher compared to the breastfed group ($p = 0.012$) (**Table 4**).

Z-scores for body length did not differ significantly among the formula-fed groups at any time point (**Figure 2B** and **Table 3**). The SF and MFGM groups did not differ significantly from the breastfed group at any time point, but the F19 infants had significantly greater length at ages 9 ($p = 0.009$) and 12 months ($p = 0.048$). Gain in body length (cm/day) did not differ among any of the groups during or after the intervention (**Table 4**).

Head circumference z-scores did not differ significantly among the formula-fed groups at any time between 1 and 12 months (**Figure 2C** and **Table 3**). During the intervention, the breastfed group had larger head circumference than all of the formula-fed groups at 1 and 2 months (MFGM, $p = 0.012$ and $p = 0.048$, respectively; F19, $p = 0.051$ and 0.042, respectively; SF, $p = 0.003$ and 0.024, respectively). After the intervention, the groups showed no differences in head circumference or in gains in head circumferences (**Table 4**).

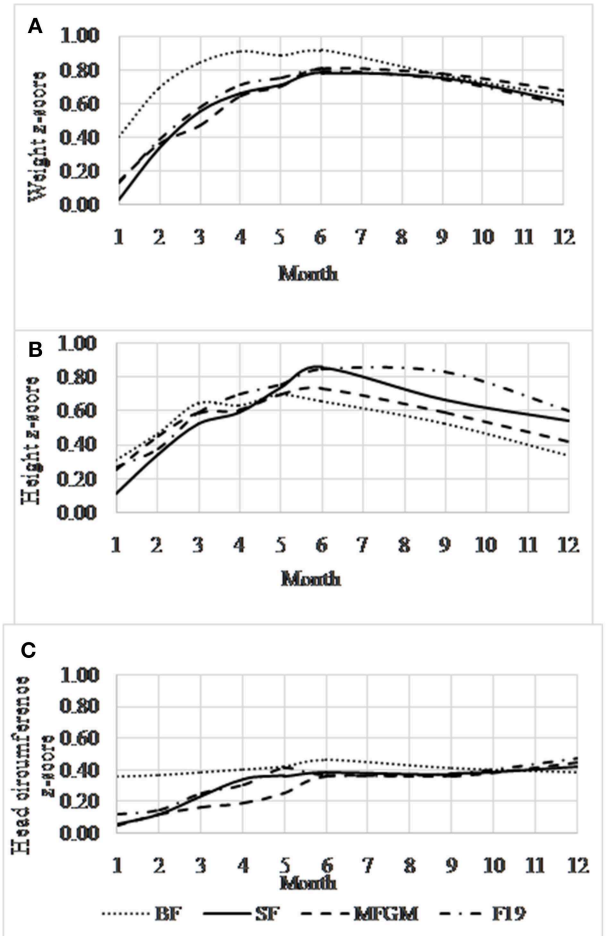

FIGURE 2 | Mean (95% confidence interval) age-adjusted anthropometric z-score data (y-axis) for the formula-fed and breastfed groups. Weight for age **(A)**, length for age **(B)**, and head circumference for age **(C)** using the World Health Organization reference population (31). BF, breastfed; SF, standard formula; MFGM, formula supplemented with milk fat globule membrane; F19, formula supplemented with *L. paracasei* ssp. *paracasei* strain (F19). There were no significant differences among the formula-fed groups in mean z-scores at any time point or for any of the growth variables.

Primary Outcomes

During the intervention, both the MFGM and the F19 groups had numerically fewer episodes of fever (>38°C) and days with fever than the SF group, although the differences did not reach statistical significance. However, compared to the breastfed group, the SF infants had significantly more fever episodes ($p = 0.021$) and days with fever ($p = 0.036$), but not episodes of diarrhea. In contrast, neither the MFGM nor the F19 groups had significantly more episodes of fever or number of days with fever than the breastfed group (**Table 5**).

TABLE 3 | Weight, height and head circumference from 1 to 12 months of age.

	BF	SF	MFGM	F19	SF vs. MFGM	SF vs. F19
	(*n* = 208)	(*n* = 194)	(*n* = 192)	(*n* = 195)	*p*-values	*p*-values
WEIGHT (KG)						
1 month	4.6	4.4	4.4	4.4	0.612	0.532
2 months	5.8	5.6	5.6	5.7	0.999	0.746
3 months	6.8	6.6	6.5	6.6	0.836	0.999
4 months	7.5	7.3	7.2	7.3	0.999	0.999
5 months	8.0	7.8	7.8	7.8	0.999	0.999
6 months	8.4	8.4	8.4	8.4	0.999	0.999
9 months	9.4	9.4	9.4	9.4	0.999	0.999
12 months	10.2	10.1	10.2	10.1	0.900	0.999
HEIGHT (CM)						
1 month	54.8	54.4	54.7	54.7	0.432	0.310
2 months	58.7	58.5	58.6	58.5	0.828	0.999
3 months	61.9	61.7	61.8	61.8	0.999	0.999
4 months	64.3	64.3	64.2	64.5	0.999	0.780
5 months	66.4	66.6	66.4	66.6	0.999	0.999
6 months	68.1	68.6	68.3	68.5	0.390	0.999
9 months	72.2	72.6	72.4	73.0	0.848	0.240
12 months	75.7	76.2	75.9	76.4	0.472	0.999
HEAD CIRCUMFERENCE (CM)						
1 month	37.3	37.0	37.0	37.0	0.999	0.999
2 months	39.1	38.8	38.8	38.9	0.999	0.999
3 months	40.4	40.3	40.2	40.3	0.802	0.999
4 months	41.6	41.5	41.3	41.4	0.292	0.590
5 months	42.5	42.5	42.4	42.5	0.999	0.999
6 months	43.3	43.2	43.2	43.2	0.956	0.999
9 months	44.9	44.9	44.8	44.9	0.999	0.999
12 months	46.0	46.1	46.1	46.1	0.999	0.999

BF, breastfed reference group; SF, standard formula; MFGM, formula supplemented with milk fat globule membrane; F19, formula supplemented with Lactobacillus paracasei ssp. paracasei strain F19.

During the intervention, the number of episodes of upper respiratory tract infections did not differ among the formula-fed groups or between these groups overall and breastfed infants; however, the F19 group had more episodes than the breastfed group ($p = 0.048$). The F19 infants also had more episodes of lower respiratory tract infections than the breastfed group ($p = 0.009$).

During the post-intervention period (5–12 months), the formula-fed groups did not differ significantly for any of the primary outcomes except for marginally more episodes of upper respiratory tract infections for the MFGM infants compared to the SF group ($p = 0.050$). Compared to the breastfed infants, the MFGM group had more episodes of diarrhea ($p = 0.021$) (**Table 5**). Per-protocol analyses of the primary outcomes did not differ from the intention-to-treat analyses (data not shown).

Secondary Outcomes

During the intervention, the formula-fed groups did not differ with respect to skin affections, use of antibiotics, vomiting, or unscheduled visits/hospitalizations (**Table 6**). However, compared to the breastfed group, the F19 infants used significantly more antibiotics and had more unscheduled visits and/or hospitalization episodes ($p = 0.045$ and 0.015, respectively). After the intervention, only the F19 group used more antibiotics compared to the breastfed infants ($p = 0.003$), with no difference among the formula-fed groups. None of the formula-fed infants had more unscheduled visits/hospitalizations than the breastfed group after the intervention.

Adverse Events

The number of AEs was low, with no differences within any of the reported categories among the formula-fed groups or between breastfed and formula-fed infants during the intervention or the post-intervention period (**Table 7**). However, during the intervention, the total number of AEs was highest in the SF group and significantly higher than in the F19 group ($p = 0.046$), but with no significant difference after the intervention.

TABLE 4 | Mean weight/length/head circumference gain during 0–4 months and 5–12 months.

	BF	SF	MFGM	F19	SF vs. MFGM	SF vs. F19
	(*n* = 208)	(*n* = 194)	(*n* = 192)	(*n* = 195)	*p*-values	*p*-values
0–4 MONTHS						
Weight gain (g/day)	31.5	31.7	30.9	31.7	0.508	0.999
Length gain (cm/day)	0.104	0.107	0.104	0.106	0.276	0.999
Head circumference (cm/day)	0.047	0.050	0.048	0.047	0.698	0.912
5–12 MONTHS						
Weight gain (g/day)	10.3	10.8	11.4	10.9	0.224	0.999
Length gain (cm/day)	0.044	0.045	0.044	0.046	0.656	0.999
Head circumference (cm/day)	0.017	0.017	0.017	0.017	0.112	0.999

BF, breastfed reference group; SF, standard formula; MFGM, formula supplemented with milk fat globule membrane; F19, formula supplemented with Lactobacillus paracasei ssp. paracasei strain F19.

TABLE 5 | Primary outcomes during 0–4 months and 5–12 months.

	BF	SF	MFGM	F19	SF vs. MFGM	SF vs. F19
	(*n* = 208)	(*n* = 194)	(*n* = 192)	(*n* = 195)	*p*-values	*p*-values
0–4 MONTHS						
Diarrhea (episodes)	14	9	7	15	0.999	0.532
Fever > 38°C (episodes)	6	21	11	10	0.242	0.214
Days with fever	8	31	18	11	0.230	0.122
Other infections (episodes)						
URI	18	27	28	33	0.999	0.966
LRI	0	5	2	8	0.898	0.999
5–12 MONTHS						
Diarrhea (episodes)	25	34	45	36	0.422	0.999
Fever >38°C (episodes)	77	85	94	79	0.908	0.999
Days with fever	179	197	224	185	0.999	0.999
Other infections (episodes)						
URI	82	82	104	101	0.050	0.136
LRI	3	5	3	8	0.999	0.999

BF, breastfed reference group; SF, standard formula; MFGM, formula supplemented with milk fat globule membrane; F19, formula supplemented with Lactobacillus paracasei ssp. paracasei strain F19; URI, upper respiratory tract infection; LRI, lower respiratory tract infection.

In total, 20 infants had SAEs, and one infant had two events. The SAEs were distributed among the groups as follows: breastfed, four; SF, four; MFGM, seven; and F19, six. The most common SAE was lower respiratory tract infection, with 16 episodes. During the intervention period, there were six SAEs, three among breastfed infants, one in the MFGM group, and two in the F19 group.

Of all the reported AEs and SAEs, 12 were considered probably related to the formula and one definitely related by the responsible pediatrician. Eleven of the infants developed skin affections and one infant constipation. Four of the infants had no treatment for their skin affections, five had local treatment with lubricant and/or steroids, and one infant also local antibiotics. The formula was switched to another formula by the parents of one infant (constipation), and 3 days later this infant dropped out from the study. For another infant, the formula was switched to a partially hydrolyzed formula. This infant had a skin infection and was treated with local steroids and antibiotics. Whether the switch of formula had an effect is unknown. For a third infant, formula was also withdrawn, and the mother went back to exclusive breastfeeding. The only infant for whom the skin affection was classified as definitely related to the formula stopped eating the formula, but the diagnosis was never proven by challenging the infant with the formula. This infant belonged to the MFGM group. The 12 infants experiencing these events were distributed across all groups: SF, four; F19, four; MFGM, two; and breastfed, two. Thus, the AEs possibly related to the formula were few and not proven in any of the infants, and there was no significant difference among the groups.

TABLE 6 | Secondary outcomes during 0–4 months and 5–12 months.

	BF	SF	MFGM	F19	SF vs. MFGM	SF vs. F19
	(*n* = 208)	(*n* = 194)	(*n* = 192)	(*n* = 195)	*p*-values	*p*-values
0–4 MONTHS						
Skin effects	17	20	16	16	0.999	0.980
Use of antibiotics	7	21	9	19	0.128	0.999
Vomiting	0	0	1	0	–	–
Unscheduled visits and/or hospitalization	9	23	15	25	0.986	0.798
5–12 MONTHS						
Skin infections	4	0	2	0	0.480	–
Use of antibiotics	33	50	53	65	0.999	0.506
Vomiting	0	1	0	0	–	–
Unscheduled visits and/or hospitalization	45	54	55	70	0.720	0.708

BF, breastfed reference group; SF, standard formula; MFGM, formula supplemented with milk fat globule membrane; F19, formula supplemented with Lactobacillus paracasei ssp. paracasei strain F19.

S-Ferritin

S-ferritin concentration was analyzed in a random sample consisting of 50 infants from each of the groups (**Figure 3**). There was no difference in s-ferritin results among the formula-fed groups, but values in the F19 group were significantly lower than in the breastfed group ($p = 0.008$). The SF and MFGM groups did not differ from the breastfed infants. There was a sex difference in s-ferritin in all groups, with girls having higher values, and values for girls differed significantly from boys for the breastfed and F19 groups. The number of infants with iron deficiency, defined as s-ferritin <12 μg/L, was two each in the breastfed and F19 groups and one each in the SF and MFGM groups.

DISCUSSION

In this randomized, double-blind, controlled multicenter study, we evaluated the safety and effects on infections and growth of two infant formulas, one supplemented with the probiotic bacterium *L. paracasei,* ssp. *paracasei* strain F19 and the other with the bovine MFGM fraction Lacprodan® MFGM-10, as compared to the same unsupplemented standard infant formula. A breastfed group served as reference.

All three formulas were well accepted by the infants as well as the parents/care providers. Although the F19 group consumed slightly less formula per day than the SF group, all formula-fed groups had a higher average intake of formula during the intervention than recommended by the manufacturer, suggesting satisfactory adherence. This inference is further supported by the fact that of the 100 infants randomized in each group at 4 months, 92% of the F19 infants had detectable F19 in their stool, compared with none in the other two groups (data not shown).

Growth is an important safety outcome for any new ingredient used in infant formula (34). The formula-fed groups showed no difference from each other in weight, length, or head circumference z-scores at any time point. Compared with the breastfed reference group, at entry, infants assigned to the formula-fed groups were smaller, particularly with lower weight because of lower birth weight. This baseline difference is a reasonable explanation for why particularly early anthropometric measures (1–4 months) were higher for the breastfed group than for the formula-fed groups. A higher weight among breastfed infants compared to formula-fed infants early in life has been described in many studies (35, 36), as has also that this difference disappears after age 4–6 months. Average daily gain in weight, length, or head circumference did not differ among the formula-fed groups or between these infants overall and the breastfed group during the intervention. A previous report indicated that providing F19 during the weaning period does not affect body composition, growth, or any of the assessed metabolic markers at school age (37), and another study showed that supplementing an infant formula with the same MFGM fraction as used here did not affect growth (29). Overall, the anthropometric data for the MFGM and F19 groups did not differ from those of the SF group, taking the difference in size at birth into consideration. All three formula-fed groups showed growth patterns similar to formula-fed infants in other published studies and tracked with the World Health Organization growth charts.

We identified few AEs overall, with no differences within any of the AE categories among the formula-fed groups or between the breastfed and formula-fed groups during the intervention or the post-intervention period. The total number of AEs during the intervention was numerically higher among SF infants than in other groups, significantly so compared to the F19 group, but no groups differed after the intervention. Only 20 infants experienced SAEs, most commonly lower respiratory tract infections, with 16 episodes. Taking AEs and SAEs together, in no case could it be definitely concluded that the formula was the cause. A recent study from China from one of the study sites showed low iron status in both breastfed and formula-fed infants (38). In the present study, we found very few infants with iron deficiency, and overall iron status was satisfactory in all groups. The reason for this discrepancy is not known, but many factors

TABLE 7 | Adverse events during 0–4 months and 5–12 months.

	BF	SF	MFGM	F19	SF vs. MFGM	SF vs. F19
	(*n* = 208)	(*n* = 194)	(*n* = 192)	(*n* = 195)	*p*-values	*p*-values
0–4 MONTHS						
Oral infections	1	2	1	1		
Gastrointestinal infections[a]	1	4	3	0		
Other viral infections	1	0	0	0		
Other bacterial infections	0	0	1	0		
Hematochezia	0	2	0	0		
Constipation	1	2	1	0		
Other non-infectious diseases	0	1	0	0		
Skin effects	1	4	1	4		
Total	5	15	7	5	0.140	0.046
5–12 MONTHS						
Oral infections	5	2	0	2		
Gastrointestinal infection[a]	0	1	0	0		
Other viral infections	5	4	8	3		
Other bacterial infections	2	0	0	1		
Hematochezia	0	0	0	0		
Constipation	0	0	0	0		
Other non-infectious diseases	0	0	1	2		
Skin effects	1	0	0	0		
Total	13	7	9	8	0.999	0.999

BF, breastfed reference group; SF, standard formula; MFGM, formula supplemented with milk fat globule membrane; F19, formula supplemented with Lactobacillus paracasei ssp. paracasei strain F19. [a]Not diarrhea.

affect iron status during early infancy including maternal iron status, cord clamping (39), and type of feeding (40).

Several randomized double-blind trials have assessed the effects on health of adding MFGM to infant formulas or diets for young children. In Belgian preschool children, a daily chocolate formula-milk supplemented with a phospholipid-rich MFGM concentrate resulted in a significantly reduced number of days with fever during the 4-month intervention period compared to the corresponding unsupplemented formula-milk (41). In a Peruvian double-blind randomized controlled trial healthy, primarily breastfed infants ages 6–11 months were given instant complementary food fortified with 1 RDA of multiple micronutrients, with either an MFGM-enriched protein fraction or skim milk powder (control group) as the protein source, daily for 6 months. The primary outcome was diarrhea. The groups showed no difference in the incidence of diarrhea, although the longitudinal prevalence of diarrhea was significantly lower in the MFGM compared with the control group. In a multivariate model adjusted for initial anemia and potable water facilities, the incidence of bloody diarrhea was lower in the MFGM group (30). In a Swedish study, term infants were randomized before the age of 2 months to formula with slightly reduced protein and energy content and supplemented with the same MFGM preparation or SF (28, 42, 43). A breastfed group served as reference. The formulas were used until age 6 months, and the infants were followed to age 12 months. During the intervention, the MFGM group had a lower incidence of acute otitis media than the SF group (1% vs. 9%, $p = 0.034$), lower incidence and longitudinal prevalence of antipyretic use, and a lower concentration of secretory IgG against pneumococci after vaccination (29), in agreement with previous findings of reduced infections.

In contrast, in a multicenter non-inferiority DBRCT on healthy term infants, Billeaud et al. evaluated the safety of two infant formulas, enriched with a lipid-rich or a protein-rich bovine MFGM fraction, respectively. At 14 days of age, the infants were randomized to receive standard infant formula (control), or one of the two experimental formulas until age 4 months. The primary outcome, weight gain, was non-inferior in the MFGM-lipid and MFGM-protein groups compared with the control group. Among secondary and exploratory outcomes, few between-group differences were observed. AEs and morbidity rates were similar across groups except for a higher rate of eczema with protein-rich MFGM compared to the other two groups (44). Of note, however, the total number of infants with eczema was low, and a Swedish study did not have a similar finding (45). A trial in India, evaluating the preventive effect against diarrhea of supplementation with a ganglioside concentrate during the second year of life, was inconclusive in the primary outcome of rotavirus diarrhea, and in secondary outcomes, including all-cause diarrhea (46). However, the study was underpowered because of a lower-than-expected diarrhea incidence.

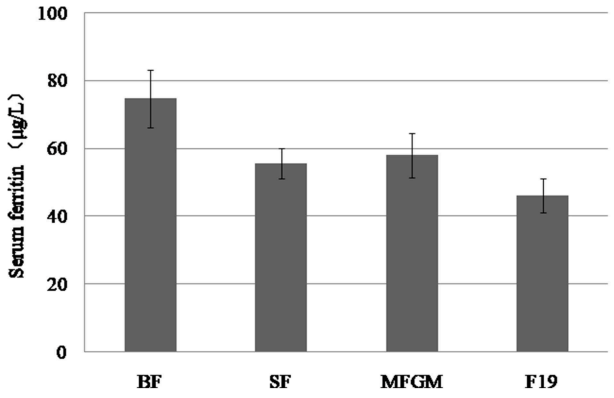

FIGURE 3 | Serum ferritin levels in the breastfed group and in the formula-fed groups. S-ferritin concentration was analyzed in a random sample consisting of 50 infants from each of the four groups. There was no difference in s-ferritin concentration among the formula-fed groups, but the F19 group was significantly lower than the breastfed group ($p = 0.008$), while there was no difference between the standard formula and MFGM and the breastfed group. There was a sex difference, with s-ferritin being higher in girls than in boys, a difference that was statistically significant for the breastfed and F19 groups. Error bars: 95% confidence intervals.

In our study, compared to the breastfed group, the SF but not the MFGM and F19 infants had significantly more episodes of fever and days with fever during the intervention. Numerically the MFGM and F19 groups also had fewer episodes of fever and days with fever than the SF group, but not significantly. During the intervention, number of episodes of respiratory tract infections did not differ among the formula-fed groups or between these infants overall and the breastfed group; however, F19 infants had more episodes of upper and lower respiratory tract infections compared to the breastfed group. Furthermore, during the intervention, formula-fed infants did not differ among groups for skin affections, vomiting, or unscheduled visits/hospitalizations, although the F19 group used more antibiotics and had more unscheduled visits/hospitalizations than the breastfed group. After the intervention, only the F19 infants used more antibiotics than the breastfed group, while formula-fed groups showed no differences.

Of interest, during the intervention, the MFGM group did not have significantly more episodes of fever or number of days with fever, diarrhea, and use of antibiotics or unscheduled visits/hospitalization during the intervention than the breastfed group. This finding suggests health benefits particularly for this group. However, these positive effects were less obvious after the intervention when the MFGM group had more episodes of diarrhea than the breastfed infants. The incidence of diarrhea during the study period was lower than expected, making the study underpowered compared with the intention of the design. Furthermore, otitis media was not diagnosed because otoscopy check is not a clinical routine, nor was cognitive development assessed in this study. These results support the previous observation that supplementation with MFGM reduces the gap between formula-fed and breastfed infants with regard to infections. Preclinical studies have demonstrated that several proteins in the MFGM inhibit various pathogens, including *Escherichia coli*, rotavirus, and enterotoxins (25, 47–49). Further studies are needed to clarify the mechanisms behind the anti-infectious properties of MFGM.

Probiotics have been proposed to influence a wide range of health outcomes, presumably by altering the intestinal microbiota and, directly or indirectly, modulating the developing immune system (7). Studies on the probiotic F19 during the weaning period have shown a lower incidence of eczema at age 12 months (19) and increased capacity to raise immune responses to protein antigens (50). Another study, comparing infant formulas containing oligosaccharides with or without F19, showed a lower incidence of lower respiratory tract infections with synbiotics compared to prebiotics (20). In the present study, we found no significant difference in stool frequency, stool consistency (data not shown), or diarrhea episodes between the F19 and SF groups during the intervention. The two groups also did not differ for other primary or secondary outcomes. However, compared to the breastfed group, the F19 infants had more upper and lower respiratory tract episodes, as noted, along with use of antibiotics, and unscheduled visits/hospitalizations during the intervention period and more use of antibiotics after the intervention. Given the previous observation of lower frequency of lower respiratory tract infections in infants given F19 together with prebiotics (20) and less antibiotic use in infants fed F19 (50), the present observations are difficult to explain. However, besides these unexpected findings, overall, we observed no negative effects of adding probiotics (*Lactobacillus* F19) to infant formula, in agreement with previous studies. Of interest, consumption of probiotics during early infancy and increased infection risk among toddlers has been demonstrated (51), although the evidence is not conclusive.

Strengths of the present study are the large number of infants included and the double-blind randomized controlled design. Both the F19- and MFGM-enriched formulas met the primary safety endpoint with respect to anthropometrics compared to the SF group and also the breastfed reference group. In general, the formulas were well-tolerated with few AEs. The limitations of the study for investigating other outcomes include the number of sites, the absence of otitis media assessment, and the lack of cognitive development screening.

CONCLUSIONS

Both the MFGM- and F19-supplemented formulas met the primary safety endpoint of weight gain that did not differ from infants assigned to the control formula. In general, the formulas were well-tolerated but showed no obvious positive effects on the health outcomes studied. Of note, however, during the intervention, the outcomes for the MFGM group were close to those of the breastfed group, supporting previous findings showing that supplementing infant formulas with MFGM narrows the gap between breastfed and formula-fed infants with respect to infections. Our findings provide support for further clinical evaluation of MFGM- or F19-enriched infant formulas.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript/supplementary files.

AUTHOR CONTRIBUTIONS

XL, YP, BL, and OH: conceptualization. XL, YP, ZL, BL, and OH: principal investigators. HS: statistical analyses. XL, YP, BL, OH, and HS: methodology. XL, YP, OH, and BL: supervision. HS: visualization. XL, OH, HS, and BL: writing, original draft. XL, YP, ZL, BL, AH, HS, BC, and OH: writing, review and editing.

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REVIEW
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Small Intestinal Bacterial Overgrowth in Children: A State-Of-The-Art Review

David Avelar Rodriguez[1], Paul MacDaragh Ryan[2], Erick Manuel Toro Monjaraz[1], Jaime Alfonso Ramirez Mayans[1] and Eamonn Martin Quigley[3]*

[1] Pediatric Gastroenterology and Nutrition Unit, National Institute of Pediatrics, Mexico City, Mexico, [2] School of Medicine, University College Cork, Cork, Ireland, [3] Lynda K. and David M. Underwood Center for Digestive Disorders, Houston Methodist Hospital, Houston, TX, United States

Small intestinal bacterial overgrowth (SIBO) is a heterogenous and poorly understood entity characterised by an excessive growth of select microorganisms within the small intestine. This excessive bacterial biomass, in turn, disrupts host physiology in a myriad of ways, leading to gastrointestinal and non-gastrointestinal symptoms and complications. SIBO is a common cause of non-specific gastrointestinal symptoms in children, such as chronic abdominal pain, abdominal distention, diarrhoea, and flatulence, amongst others. In addition, it has recently been implicated in the pathophysiology of stunting, a disease that affects millions of children worldwide. Risk factors such as acid-suppressive therapies, alterations in gastrointestinal motility and anatomy, as well as impoverished conditions, have been shown to predispose children to SIBO. SIBO can be diagnosed via culture-dependant or culture-independent approaches. SIBO's epidemiology is limited due to the lack of uniformity and consensus of its diagnostic criteria, as well as the paucity of literature available. Antibiotics remain the first-line treatment option for SIBO, although emerging modalities such as probiotics and diet manipulation could also have a role. Herein, we present a state-of-the-art-review which aims to comprehensively outline the most current information on SIBO in children, with particular emphasis on the gut microbiota.

Keywords: small intestine bacterial overgrowth, small intestinal bacterial overgrowth, small bowel bacterial overgrowth, proton pump inhibitors, gut microbiota, stunting

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****Correspondence:***
David Avelar Rodriguez
davidavelar1@outlook.com

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INTRODUCTION

Small intestinal bacterial overgrowth (SIBO) is a heterogenous disorder characterised by an excessive growth of select microorganisms within the small intestine. This excessive bacterial biomass, in turn, disrupts host physiology in a myriad of ways, leading to gastrointestinal and non-gastrointestinal symptoms and complications (1). SIBO is a common cause of non-specific gastrointestinal symptoms in children, such as chronic abdominal pain, abdominal distention, diarrhoea, and flatulence, amongst others (2–5). In addition, it has recently been implicated in the pathophysiology of stunting (6), a disease that affects millions of children worldwide. Certain risk factors, such as acid-suppressive therapies (7–10), alterations in gastrointestinal motility and anatomy (11–20), and impoverished conditions (21–26), have been shown to predispose children to SIBO. Despite the relatively high prevalence of SIBO in children, it remains a poorly understood

disorder. In fact, only a small number of studies have been published in the last two decades. Since the year 2000, only 149 articles have been published on paediatric SIBO (**Figure 1**). Due to this previous scarcity of literature and the recent emergence of novel, informative data regarding the underpinnings of the disease, an update for practicing paediatricians is now warranted. This article represents a state-of-the-art review which aims to comprehensively outline the most current information on SIBO in children, with particular emphasis on the gut microbiota.

SEARCH STRATEGY AND SELECTION CRITERIA

In December 2018, two investigators systematically searched the PubMed/MEDLINE, EBSCOhost, Google Scholar, and ResearchGate databases using the primary search blocks "small intestine bacterial overgrowth OR small intestinal bacterial overgrowth OR small bowel bacterial overgrowth AND children" and "small intestine bacterial overgrowth OR small intestinal bacterial overgrowth OR small bowel bacterial overgrowth." In addition, the following terms were used in combination: "small intestinal bacterial overgrowth," "pathophysiology," "16S rRNA sequencing," "next generation sequencing," "gut microbiota," "proton pump inhibitors," "juvenile systemic sclerosis," "scleroderma," "ileocecal valve," "vitamin," "functional gastrointestinal disorders," "chronic abdominal pain," "irritable bowel syndrome," "constipation," "hydrogen," "methane," "breath test," "stunted," "environmental enteric dysfunction," "environmental enteropathy," "*Methanobrevibacter smithii*," "methanogens," "immunodeficiency," and "coeliac disease." The search was restricted to titles and abstracts written in English and Spanish, and no date range restriction was applied. We also searched for ongoing clinical trials in Clinicaltrials.gov with the term "small intestinal bacterial overgrowth." Adult patient studies were included when relevant. In addition, we screened the reference lists of the selected papers and created weekly alerts on PubMed to enhance the sensitivity of the search. The last literature search was conducted on May 13th, 2019.

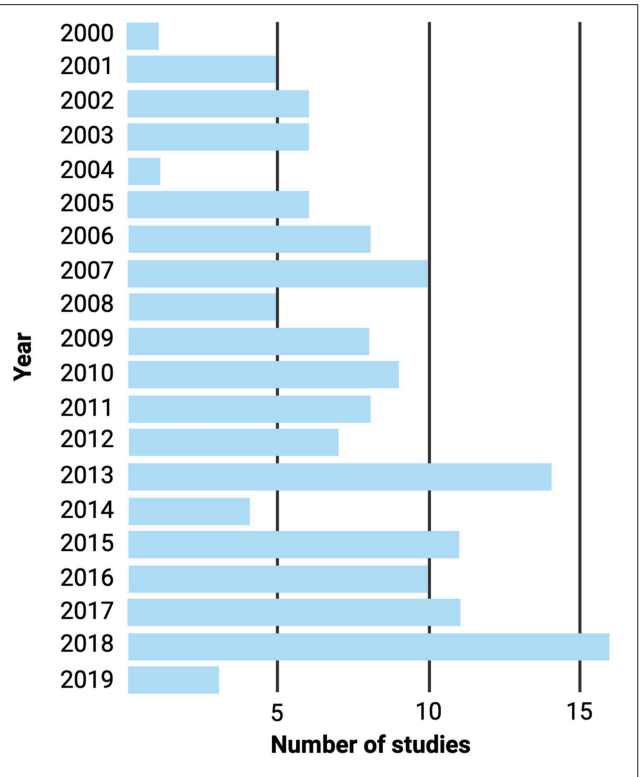

FIGURE 1 | Number of paediatric SIBO publications on PubMed from 2000 to 2019. PubMed literature search with the entry block "(children OR pediatrics) AND (small intestinal bacterial overgrowth OR small intestine bacterial overgrowth OR small bowel bacterial overgrowth)"; the search was conducted on March 26, 2019.

THE GASTROINTESTINAL TRACT AND THE GUT MICROBIOTA

Each of the organs and anatomical portions of the gastrointestinal system provides specific functions and are equipped with mechanisms to promote a homeostatic relationship with its indigenous bacterial community (microbiota) (27, 28). This mutualistic homeostasis is achieved through mechanisms including gastric and bile acids, pancreatic and digestive secretions, intestinal motility, the barrier function of the ileocecal valve, amongst others. Within the lumen of the gastrointestinal tract resides the gut microbiota, a complex and susceptible microbial consortium composed of enteric-adapted bacteria, which is acquired in the very early stages of life and develops in concert with the host. The "sterile womb hypothesis" refers to the notion that the neonate acquires the gut microbiota during birth through ingestion of the mother's vaginal (vaginal delivery) or skin (caesarean section) microbiota. In contrast, although increasing evidence has suggested that the infant gut microbiota is acquired *in utero*, a technically comprehensive multimodal investigation recently failed to detect any placenta dwelling microorganisms (29). The gut microbiota maintains a life-long symbiotic relationship with the human host, providing a plethora of important functions; from vitamin and short-chain fatty acid (SCFA) production to immunoregulation and neuropeptide secretion [for review see (30)].

The composition and functionality of the gut microbiota is shaped and influenced by a multitude of intrinsic and extrinsic factors, such as genetics, mode of delivery, gestational age, feeding type and diet, pharmaceuticals, and exercise (30). The composition of the gut microbiota exhibits variations related to the anatomical portion studied, which is mainly influenced by factors such as the luminal oxygen concentration and pH. Indeed, bacterial numbers increase from about 10^3 to 10^4/ml in the stomach to approximately 10^{11}/ml in the colon (**Figure 2**) (31–33). The colonic microbiota is the largest prokaryotic community in the human body, representing nearly the 0.3% of the overall host body weight (34). Although composed largely of four bacterial phyla – Actinobacteria, Firmicutes, Bacteroidetes, and Proteobacteria–

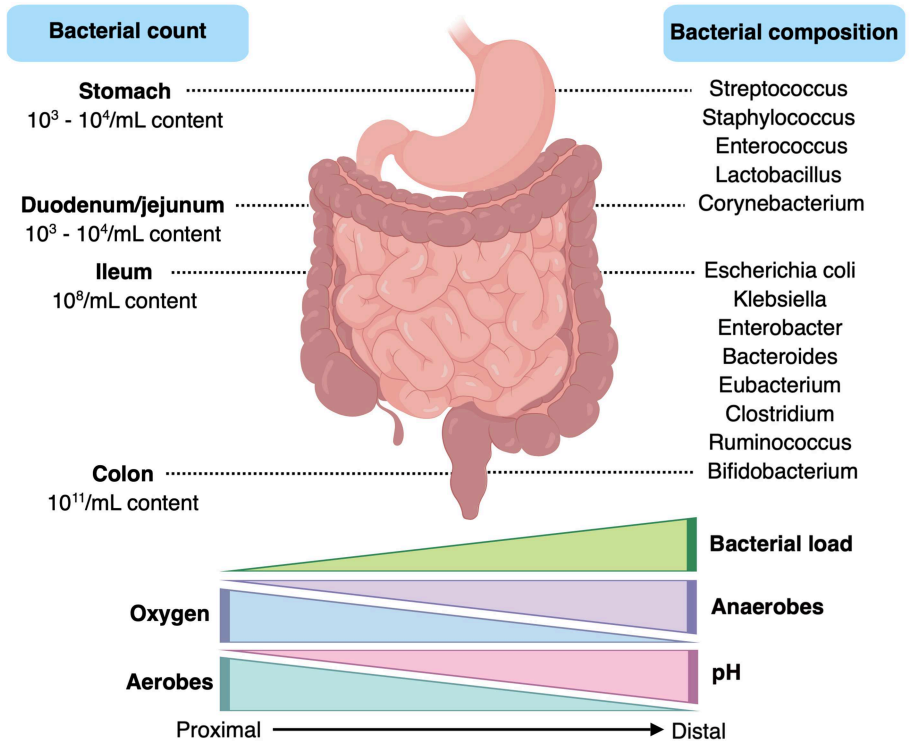

FIGURE 2 | Gastrointestinal tract features and bacterial composition and content. Created with BioRender.com.

the infantile gut microbiome is dominated by Actinobacteria, with Firmicutes emerging to dominate after infancy (35). In general, bacteria from the Actinobacteria (e.g., *Bifidobacterium*), Firmicutes (e.g., *Faecalibacterium*, *Clostridium, Ruminococcus, Lactobacillus*) and Bacteroidetes (e.g., *Bacteroides, Prevotella*) phyla are largely regarded as commensal microorganisms, while a significant portion of gastrointestinal pathogens belong to the Proteobacteria phylum (e.g., *Escherichia, Shigella, Salmonella, Klebsiella,* and *Helicobacter,* amongst others) (36, 37). In addition to this diverse community of bacteria, the human gastrointestinal tract is also home to an extensive array of viruses (38), fungi (39), and archaea (40). While it remains unclear at present as to whether the former two kingdoms play a significant role in SIBO pathogenesis, methanogenic archaea, such as *Methanobrevibacter* spp., have been directly implicated in a methane-specific form of the disorder (41).

It is important to note, however, that despite the substantial advances made in the study of the gut microbiota in general, the small bowel microbiota remains poorly understood (42). Whereas the colonic microbiota is more easily accessible and can be sampled via colonoscopy or faecal sample (43), sampling of the small bowel microbiota poses a major challenge due to the invasiveness of the procedures (upper endoscopy) and the technical difficulties associated with these. Therefore, this significantly obscures our understanding of SIBO and hampers the establishment of a definition.

THE EPIDEMIOLOGY OF SIBO IN CHILDREN

The prevalence of SIBO in children has been explored in a wide spectrum of clinical contexts, including children living in impoverished conditions, individuals with chronic abdominal pain (CAP), as well as those who suffer from irritable bowel syndrome (IBS), stunting, and obesity, amongst other diseases. SIBO prevalence ranges from about 9% in children taking proton pump inhibitors (PPIs) (7) to approximately 90% in those with stunted growth (6) and chronic abdominal pain (CAP) (2). However, it should be noted that the data on the epidemiology of SIBO in children is limited by the small number of studies available, the lack of appropriate controls in some studies, and the varying test methodology and diagnostic cut-offs applied. **Table 1** shows the study characteristics and reported SIBO prevalence in children with a wide variety of clinical contexts and risk factors.

SIBO PATHOGENESIS

SIBO can negatively impact the host in a range of ways (1, 31, 53–61). These include bacterial carbohydrate fermentation leading to excess gas and water production (1); bacterial deconjugation of bile acids resulting in poorly absorbed liposoluble vitamins (18); bacterial macronutrient and micronutrient consumption (bacterial-host nutrient competition), leaving the host with less available nutrients for absorption (58); villous blunting leading to carbohydrate malabsorption (13, 62–64); decreased short

TABLE 1 | Study characteristics and reported SIBO prevalence in children with a wide variety of clinical contexts and risk factors.

References	Year	Study population	Study design	Sample size (n)	Diagnostic tests and criteria for positivity	Reported SIBO prevalence*
Pignata et al. (44)	1990	Children with immunodeficiency syndromes aged 2 to 17 years and age-matched control children.	Cross-sectional	Cases: 17 Controls: 10	Jejunal aspirate cultures (not performed in controls due to ethical reasons). Positivity was defined as $\geq 10^5$ CFU/ml. GHBT Cut-off point was defined as 10 ppm.	Jejunal aspirate: Cases: 41%
Pereira et al. (21)	1991	Children under the age of 5 years living a rural village in Myanmar.	Cross-sectional	Cases: 340	LHBT Positivity was defined as "a transient breath hydrogen peak at the 20, 40, or 60 min breath samples following the lactulose test meal, and distinguishable from the later colonic peak".	27.2%
de Boissieu et al. (3)	1996	Children with chronic diarrhoea, abdominal pain, or both, aged 2 months to 12 years.	Prospective	Cases: 53	GHBT. Positivity was defined as a H_2 value $\geq$10 ppm over baseline after ingestion of glucose.	34%
Lewindon et al. (13)	1998	Children with cystic fibrosis and non-cystic fibrosis children (controls).	Cross-sectional	Cases: 19 Controls: 508	LHBT Positivity was not specified.	Cases: 32% Controls: 7% *P:* < 0.003
Fontanele Soares et al. (20)	2005	Children with chronic constipation aged 3 to 13 years.	Cross-sectional	Cases: 40	CH_4 breath test Positivity (methane producers) was defined as a methane concentration < 3 ppm.	73.5%
Dos Reis et al. (22)	2007	Children living in a slum and age and sex-matched controls aged 5 to 11 years.	Cross-sectional	Cases: 50 Controls: 50	Glucose and lactulose H_2 breath tests. Positivity was defined as an increase in H_2 of $\geq$20 ppm over baseline in the initial 60 min.	Lactulose: Cases: 37.5% Controls: 2.1% *P*: <0.001
Fridge et al. (12)	2007	Children and adults with CF and pancreatic insufficiency (mean age 17 years) and age-matched controls.	Cross-sectional	Cases: 25 Controls: 25	Glucose H_2/CH_4 breath test. Positivity was defined as either a fasting H_2 $\geq$15 ppm, a rise of $\geq$10 ppm over baseline at any time during the test, or a doubling of baseline CH_4 excretion at any time during the test. CH_4 excretors were defined by a CH_4 level of >2ppm in any sample.	Cases: 56% Controls: 20% *P:* 0.02
Scarpellini et al. (45)	2009	Children with IBS (Rome II criteria) and healthy age- and sex-matched controls.	Cross-sectional	Cases: 43 Controls: 56	Lactulose H_2/CH_4 breath test. Positivity was defined as an early rise in H_2 or CH_4 excretion of >20 ppm within the first 90 min.	Cases: 65% Controls: 7% *P:* <0.00001
Lisowska et al. (11)	2009	Children with cystic fibrosis and controls with gastrointestinal symptoms aged 5 to 17 years.	Cross-sectional	Cases: 62 Controls: 390	Glucose H_2/CH_4 breath test. Positivity was defined as a fasting H_2 or CH4 level of $\geq$20 ppm and $\geq$10 ppm, respectively; or an increase in H_2 or CH4 over baseline during the test of $\geq$12 pm and $\geq$6, respectively.	Cases: 37.1% Controls: 13.3% *P:* <0.00001
Collins et al. (46)	2010	Children with CAP (Rome II criteria) aged 8 to 18 years and healthy controls.	Cross-sectional	Cases: 75 Controls: 40	LHBT. Positivity was defined as a rise in H_2 >20 ppm before the first 90 min	Cases: 91% Controls: 35% *P:* <0.0001
Cole et al. (16)	2010	Children younger than 2 years with short bowel syndrome receiving enteral feeds	Prospective	Cases: 10	GHBT Positivity was defined as either an increased fasting breath $H_2 \geq 20$ ppm or increase from baseline of $\geq$10 ppm after glucose ingestion	(incidence) 50%

(Continued)

TABLE 1 | Continued

References	Year	Study population	Study design	Sample size (n)	Diagnostic tests and criteria for positivity	Reported SIBO prevalence*
Leiby et al. (15)	2010	Children with secondary retentive faecal incontinence (and radiographically diagnosed faecal impaction) aged 6 to 12 years and controls with gastrointestinal symptoms but without faecal incontience.	Cross-sectional	Cases: 50 Controls: 39	Lactulose H_2/CH_4 breath test. [n] Positivity was defined as an increase in H_2 $\geq$20 ppm or in CH_4 $\geq$10 ppm over baseline at $\leq$60 min. Patients were considered CH_4 producers if their level was more than 3 ppm at any point in the study; and high basal CH_4 was diagnosed if the baseline sample was >10 ppm.	Cases: 42% Controls: 23%
Jones et al. (47)	2011	Children with chronic diarrhoea and/or abdominal pain and/or bloating and/or irritability younger than 15 years of age.	Cross-sectional	Cases: 287	CO2-corrected H_2 and CH_4 levels Positivity was defined as an increase in H_2 >10 ppm over baseline in the initial 45 min of the test. Patients were classified as H_2 or CH_4 producers if they produced >10 ppm of these gases at any time point.	87%
Mello et al. (23)	2012	Children of poor socioeconomic conditions residing in a slum and children of socioeconomically advantaged families aged 6 to 10 years.	Cross-sectional	Cases: 85 Controls: 43	Lactulose H_2/CH_4 breath test. Positivity was defined as an increase in H_2 of $\geq$ 20 ppm or CH4 of $\geq$ 10 ppm with respect to the fasting value within the first 60 min after the ingestion of lactulose? Subjects were considered CH_4-producers when the concentration of CH_4 was $\geq$ 3.	Cases: 30.9% Controls: 2.4% *P:* 0.0007
Gutierrez et al. (17)	2012	Children with intestinal failure and refractory gastrointestinal symptoms (i.e., abdominal bloating, emesis, diarrhoea, or increased stoma output) with a median age of 5 years.	Cross-sectional	Cases: 57	Duodenal aspirate cultures. Positivity was defined as a bacterial growth of $\geq 10^5$ CFU/ml.	70%
Scarpellini et al. (48)	2013	Children with IBS (Rome II criteria). *Study objective:* to assess the effects of rifaximin treatment on SIBO prevalence and gastrointestinal symptoms in children affected by IBS.	Prospective	Cases: 50	Lactulose H_2/CH_4 breath test. Positivity was defined as an early rise in H_2 or CH_4 excretion >20 ppm within the first 90 min.	66%
Ojetti et al. (14)	2013	Children with myelomeningocele and constipation.	Cross-sectional	Cases: 18	Lactulose H_2/CH_4 breath test. Positivity was not specified.	39%
Hegar et al. (9)	2013	Children with epigastric pain and a normal baseline GHBT aged $\geq$5 years were divided into two 4-week trial groups: group 1 (cases): omeprazole plus Lacidofil® (1.9 × 109 CFU *Lactobacillus rhamnosus* R0011 and 0.1 × 109 CFU *Lactobacillus acidophillus* R0052); and group 2 (controls): omeprazole plus placebo capsule. *Study objective:* to evaluate the incidence of SIBO in children treated with omeprazole and to test whether probiotics influence the incidence.	Double-blinded, placebo-controlled randomized clinical trial	Cases: 36 Controls: 34	GHBT. Positive test was defined as an increase in H_2 of >10 ppm over baseline.	(incidence) Cases: 33% Controls: 26% *P:* 0.13

(Continued)

TABLE 1 | Continued

References	Year	Study population	Study design	Sample size (n)	Diagnostic tests and criteria for positivity	Reported SIBO prevalence*
Rosen et al. (8)	2014	Children taking acid suppressive therapy for a minimum of 4 weeks (PPIs and histamine-2 antagonists) and controls (no acid suppressive therapy) aged 1 to 18 years	Cross-sectional	Cases: 48 Controls: 51	Gastric aspirate cultures. Positivity was not clearly specified.	Cases: 46% Controls: 18% *P: 0.003*
Korterink et al. (4)	2014	Children with abdominal pain–related functional gastrointestinal disorders (AP-FGID; Rome III criteria) aged 6 to 18 years.	Prospective	Cases: 161	GHBT. Positivity was defined as fasting breath H_2 concentration ≥20 ppm or increase in H_2 ≥12 ppm over baseline value.	14.3%
Lisowska et al. (49)	2014	Children with progressive familial intrahepatic cholestasis aged 8 to 25 years.	Prospective	Cases: 26	Glucose H_2/CH_4 breath test. Positivity was defined as a high baseline value (>20 ppm for hydrogen or >10 ppm for methane) or an early increase of gas excretion (>12 ppm for hydrogen or >6 ppm for methane).	35%
Sieczkowska et al. (10)	2015	Children with histology-proven peptic esophagitis aged 3 to 18 years. *Study objective*: to evaluate whether a 3-month PPI treatment regimen induces SBBO in children and if so, to determine associated symptoms.	Prospective	Cases: 40	Glucose H_2/CH_4 breath test. Positivity was defined as an increase in H_2 of ≥10 over baseline.	22.5%
Donowitz et al. (25)	2016	Bangladeshi children from an impoverished neighbourhood aged 2 years.	Cross-sectional	Cases: 90	GHBT. Positivity was defined as an increase in H_2 of ≥12 ppm over baseline.	16.7%
Cares et al. (7)	2017	Children taking PPIs for longer than 6 months' duration and controls (no PPI treatment) aged 3 to 17 years. *Study objective:* to measure the risk for SIBO in children on chronic PPI therapy and compare, using the glucose HBT, the frequency of SIBO in children taking PPIs with those who did not.	Cross-sectional	Cases: 56 Controls: 27	Glucose H_2/CH_4 breath test. Positivity was defined as an increase in either H_2 or CH_4 of >12 ppm over baseline.	Cases: 8.9% Controls: 3.7% *P:* 0.359
Wang et al. (50)	2017	Children with autism spectrum disorder and age- and sex-matched healthy controls (age was not specified)	Cross-sectional	Cases: 310 Controls: 1240	Glucose H_2/CH_4 breath test. Positivity was defined as an increase in H_2 of ≥20 ppm or CH_4 of ≥10 ppm over baseline before the first 60 min.	Cases: 31.0% Controls: 9.3% *P:* <0.0001
Mello et al. (24)	2017	Children of low socio-economic status living in an urban slum aged 5 to 11 years	Cross-sectional	Cases: 100	Lactulose H_2/CH_4 breath test. Positivity was defined as an increase in H_2 of ≥20 ppm or CH_4 of ≥10 ppm over baseline before the first 60 min. Real-time polymerase chain reaction (results are described in aetiology section)	61.0%
Belei et al. (51)	2017	Children with overweight or obesity aged 10 to 18 years and age- and sex-matched controls	Cross-sectional	Cases: 125 Controls: 120	GHBT Positivity was defined as an increase in H_2 in two consecutive measurements of at least 15 ppm over baseline.	Cases: 37.6% Controls: 3.3%

(Continued)

TABLE 1 | Continued

References	Year	Study population	Study design	Sample size (n)	Diagnostic tests and criteria for positivity	Reported SIBO prevalence*
Galloway et al. (18)	2018	Children with intestinal failure aged 9 months to 17 years.	Prospective	Cases: 14	Duodenal aspirate culture. Positivity was defined as $\geq 10^5$ CFU/ml.	43%
Gaffar et al. (26)	2018	Children living in a disadvantaged urban community aged 12 to 18 months.	Prospective	Cases: 194	GHBT Positivity was defined as an increase in H_2 of $\geq$12 ppm over baseline measurement on any single post-glucose reading.	14.9%
Furnari et al. (52)	2018	Subjects with cystic fibrosis older than 2 years.	Randomised, case-control Trial	Cases: 79	Glucose H_2/CH_4 breath test. Positivity was defined as either a H_2 basal level of $\geq$12 ppm, a peak of H_2 excretion of $\geq$10 ppm over baseline during the test, or a CH_4 level of $\geq$12 ppm at any point time.	31.6%
Vonaesch et al. (6)	2018	Stunted children aged 2 to 5 years and healthy controls.	Cross-sectional	Cases: 46	Duodenal aspirate culture. Positivity was defined as $\geq 10^5$ CFU/ml. 16S rRNA sequencing (results are described in the text)	Cases: 96%

**A few studies also evaluated the incidence of SIBO in children (indicated in parenthesis). GHBT, glucose hydrogen breath test; LHBT, lactulose hydrogen breath test; H_2, hydrogen; CH_4, methane; CO_2, carbon dioxide; ppm, parts per million.*

chain fatty acid production (6, 65); intestinal and systemic inflammation (16, 25, 66); and increased gut permeability (**Figure 3**). It should be noted that a recent systematic review (53) and clinical study (25) found conflicting and contradictory evidence supporting effects of gut permeability.

RISK FACTORS FOR SIBO

Acid-Suppressive Therapies

Gastric acid plays a crucial role in preventing pathogens from colonizing the human alimentary tract, particularly the proximal portions (67). Several extrinsic and intrinsic factors are known to alter this natural barrier (68), with one of them being medication-induced hypochloridria, caused most frequently by proton pump inhibitors (PPIs). PPIs are commonly prescribed for the treatment of gastroesophageal reflux disease (GERD) and, sometimes, for gastroesophageal reflux (GER) in children (69). These drugs decrease gastric acid secretion by blocking the enzyme H^+/K^+-adenosine triphosphatase located in the apical membrane of parietal cells (70). It has been estimated that approximately 34% (69) of paediatric patients treated with PPIs develop adverse effects, including infectious diarrhoea, *Clostridium difficile* infection, respiratory infections, or SIBO (71–77). In addition, PPIs have also been shown to alter the faecal gut microbiota of children and adults (78, 79). In theory, the resultant acid suppression creates a more favourable environment for bacteria to overgrow –particularly Gram-positive, aerobic bacteria (31)–, leading to the development of SIBO. However, a recent observational study (6) found contradictory evidence: despite the high prevalence of SIBO seen in the patient cohort (i.e., 96%), gastric pH was found to be in the acidic range (i.e., pH 2.7). It is important to note that pH measurements were only obtained from the study group and lacked normal reference values for comparison.

To date, four studies have assessed SIBO risk in children taking acid-suppressive treatments (80). Cares et al. (7) used the glucose H_2/CH_4 breath test to determine the presence of SIBO in children taking PPIs for a prolonged time (i.e., longer than 6 months). 77% of patients took a PPI for over 12 months, and SIBO was diagnosed in 8.9% and 3.7% of PPI-subjects and controls, respectively. Moreover, Sieczkowska et al. (10) enrolled children with histology-proven peptic esophagitis in a 3-month trial of omeprazole treatment. A glucose hydrogen breath test (GHBT) was performed before and after PPI treatment, revealing that 22.5% developed SIBO as a result of the therapy. Furthermore, Rosen et al. (8) cultured the gastric fluid of children taking PPIs for at least 4 weeks and whose last dose was taken within 24 h of sampling. Compared to the control group, the PPI-group was found to have a significantly higher prevalence of gastric overgrowth (18 vs. 46%, respectively), mainly caused by potential pathogens such as *Staphylococcus* and *Streptococcus.* In a double-blind, placebo-controlled randomised clinical trial, Hegar et al. (9) randomly assigned children to one of two 4-week treatment groups: omeprazole + placebo group or omeprazole + probiotic. SIBO had been excluded at baseline in all. Following the 4-week intervention, a second GHBT indicated that SIBO was present in 33% and 26% of omeprazole/probiotic group and omeprazole/placebo group, respectively.

Two metanalyses (81, 82) of adult patients evaluated the association between PPI therapy and SIBO risk. The most recent study by Su et al. (81) included 19 observational studies with a total of 7,055 adult subjects in the analysis. Although there was a significant degree of heterogeneity amongst the studies included, after adjusting for study quality, the authors concluded that PPI

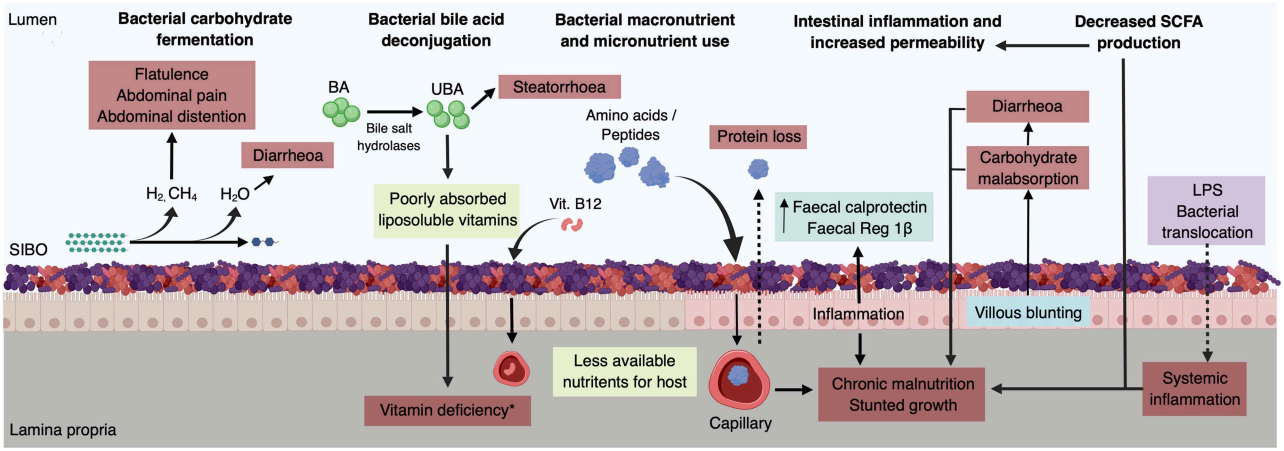

FIGURE 3 | Mechanisms through which SIBO affects the host. The dotted arrows indicate increased intestinal permeability. * Includes vitamin A, D, E, and vitamin B 12. Vitamin K is synthesised by the gut microbiota, and thus its deficiency in this context is very unlikely. BA, bile acids; UBA, unconjugated bile acids; LPS, liposaccharides. Created with BioRender.com.

therapy was associated with a moderately increased risk for SIBO (odds ratio 1.71, 95% CI 1.20–2.43). The second meta-analysis (83) included 11 studies [all of which were included in the metanalysis by Su et al. (81)] with a total of 3,134 adult subjects. Again, the authors found a statistically significant association (odds ratio, 2.282, 95% CI 1.238–4.205) between PPI therapy and SIBO, but only when diagnosis was made with "highly accurate testing modality," such as duodenal/jejunal aspirate culture. It is important to mention, however, that all the studies included in both metanalyses were observational in nature and culture or breath test-based approaches were used for SIBO diagnosis.

In conclusion, it appears that acid suppressive therapies, and in particular PPIs, seem to be a risk factor for SIBO development, but only when this is assessed by culture approaches. However, it is important to mention that the data are limited by the overall low odds ratios (∼2) and the lack of controls. Thus, until better data are available, it is of paramount importance that PPI prescriptions in children with possible GERD are judicious, and perhaps more importantly, that their use is avoided in children with GER (72, 83).

Intestinal Motility Disturbances

In addition to the acidic environment created by the gastric acid, gut motility is critical to preventing SIBO. One motor event, the migrating motor complex (MMC), a cyclic motor pattern that occurs during the interdigestive state, plays an important role in preventing the development of bacterial overgrowth within the small intestine (83). In fact, the MMC is commonly referred to as the "intestinal housekeeper," emphasising its role in gastrointestinal health (84). Under physiological conditions, the MMC sustains the aboral progression of luminal content in the small intestine between meals, thereby preventing stasis and SIBO. On this basis, an absent or disrupted MMC may lead to bacterial overgrowth (84).

The typical example of a disease with gut dysmotility leading to SIBO is systemic sclerosis, with ∼40% of adult patients being affected (54). Despite the relatively high prevalence of gastrointestinal symptoms in children with juvenile systemic sclerosis (jSS) (85), to our knowledge, no studies have investigated the association of SIBO with jSS.

A somewhat more common example of gut dysmotility associated with increased risk of SIBO is cystic fibrosis (CF) (86). Here there is limited clinical data from studies in children. Lisowska et al. (11) found a significantly higher SIBO prevalence in patients with CF compared with non-CF subjects (37 vs. 13%, respectively), and so did Fridge et al. (12) who found a significantly higher prevalence in CF-subjects than controls (56 vs. 20%, respectively). Both studies used the hydrogen breath test (HBT) for SIBO diagnosis. Moreover, Lewindon et al. (13) evaluated, with the HBT, SIBO prevalence and orocecal transit time of children with CF. Interestingly, compared with the two control groups (i.e., healthy children and non-CF patients), CF-subjects had a significantly higher SIBO prevalence and their orocecal transit times were significantly longer than those of healthy individuals. More recently, Furnari et al. (52) found a 31% SIBO prevalence in both children and adults with CF. Preclinical studies in murine models have demonstrated that CF-mice have a higher SIBO prevalence than wild-type mice (87), which is thought to be mainly caused by two factors: (1) slowed intestinal transit –possibly due to unabsorbed lipids leading to a triggering of the "ileal brake"– and/or smooth muscle dysfunction (86, 88); and (2) mucus accumulation which acts as an anchor for bacteria, thereby facilitating their overgrowth (87, 89). Thus, the multifactorial nature of CF, including gut dysmotility and impaired mucus clearance, seems to put patients at a higher risk of SIBO development.

Constipation has been shown to be associated with SIBO. However, the causative or consequential nature of this interaction is unclear. In theory, a slower orocecal transit in constipation may fail to clear the luminal content, thereby increasing SIBO risk. On the other hand, methane, a biologically active gas produced instead of hydrogen by some individuals from bacterial

fermentation of carbohydrates, can delay intestinal transit, which in turn may lead to constipation. We identified three studies that investigated the role of SIBO in children with constipation. Ojetti et al. (14) diagnosed SIBO in 39% (7/18) of children with myelomeningocele, a disease associated with constipation. Interestingly, the authors found that all methane producers had a delayed orocecal transit time. Moreover, Leiby et al. (15) found a 42% (21/50) SIBO prevalence in children with retentive faecal incontinence, of whom, eight had methanogenic SIBO, 11 had hydrogen-type SIBO, and two had mixed (methane and hydrogen)-type SIBO. In addition, 48% of patients with faecal incontience were found to have high basal methane concentrations (>10 ppm) as compared with 10% of control subjects. Furthermore, Fontanele Soares et al. (20) investigated the relationship between methane production and colonic transit time in children with constipation. Methane production was found in 73.5% (25/34) of children with constipation and soiling as compared with 1% of children with constipation alone.

The notion that a slowed intestinal transit may predispose to SIBO is supported by two recent studies in adults (90, 91). In the most recent study, by Revaiah et al. (90), two patient groups (i.e., PPI-group and PPI + prokinetic group) underwent a GHBT and lactulose hydrogen breath test (LHBT) and orocecal transit time assessment. Interestingly, SIBO was documented more frequently in the PPI alone group than in the PPI + prokinetic group, and SIBO-positive patients had slower orocecal transit times than SIBO-negative patients. However, it is important to note that the overall SIBO prevalence was only 8.8% (13 subjects) and, even though the authors measured both hydrogen and methane, it was not specified whether the three patients with methane-positive SIBO had normal or delayed orocecal transit times. Furthermore, Sarosiek et al. (91) prospectively enrolled 29 female patients with functional constipation in a 2-week lubiprostone trial. Gastrointestinal transit and SIBO were assessed by the wireless motility capsule (WMC) and lactulose hydrogen/methane breath tests, respectively. At baseline, 68% of patients had increased levels of both hydrogen and methane, suggesting SIBO. After treatment, 41% of patients became SIBO-negative, which was paralleled by a 30% increase in small bowel transit time. However, it is important to mention that the authors considered methane positivity as an increase of ≥3 ppm, which differs from the current guidelines in which methane positivity is defined as an increase of ≥10 ppm (92). These findings are difficult to interpret for two reasons: (1) all patients had constipation and were methane producers at baseline (i.e., methane production of ≥3 ppm); thus, this suggests constipation may have been caused by intestinal methane production rather than constipation leading to SIBO; or simply, the methane values present in these patients could be physiological; and (2), the fact that lubiprostone "cured" 41% of SIBO patients suggests that by increasing the intestinal transit–and thus intraluminal clearance–faecal stasis decreases, which prevents SIBO development.

Anatomical Alterations

Anatomical boundaries are crucial for maintaining a harmonized microbial community within the human alimentary tract, particularly the integrity of the ileocecal valve. Absence or dysfunction of the ileocecal valve has been shown to predispose patients to SIBO, as colonic bacteria are thought to be "backwashed" into the small intestine, thereby leading to coliform-type SIBO development (31).

Paediatric intestinal failure is a disease characterised by increased SIBO prevalence, which is thought to be due to a multifactorial process that includes altered intestinal motility and anatomy, resection of the ileocecal valve, and use of antacids (93, 94). Only four recent studies have investigated SIBO incidence/prevalence in children with intestinal failure (or short bowel syndrome) (16–19). The most recent study by Hong et al. (19) found a 78% SIBO prevalence in their patient cohort of children who underwent extensive small bowel resections due to midgut volvulus. Interestingly, only 22% of patients had a functional ileocecal valve. Moreover, Galloway et al. (18) diagnosed SIBO by duodenal aspirate cultures in 43% of patients with intestinal failure, caused mainly by gastroschisis, atresias, and necrotizing enterocolitis. Of these, only one subject had an ileocecal valve (16%). Furthermore, Gutierrez et al. (17) diagnosed SIBO in 70% of patients with intestinal failure due to various surgical and non-surgical aetiologies. Although the authors did not find a significant difference regarding SIBO prevalence between children with and without ileocecal valve, they did find a significant association between parenteral nutrition (PN) use and SIBO. The fourth study by Cole et al. (16) found a 50% SIBO incidence in their patient cohort of children with short bowel syndrome caused by necrotizing enterocolitis. Of these, 40% of children had a functional ileocecal valve. In line with the findings by Gutierrez et al. (17), another interesting study (95) that evaluated the faecal gut microbiota of children with short bowel syndrome by next generation sequencing found that children who were on PN had decreased diversity and increased numbers of Proteobacteria as compared with those who were weaned from PN. None of the PN-patients had an ileocecal valve and, of these, four out five patients were being evaluated for SIBO and were being treated with antibiotics, which may explain the microbiota perturbations observed.

Taken together, these findings demonstrate that paediatric intestinal failure is a multifactorial process that increases the risk of SIBO, as these children have a disrupted intestinal anatomy, physiology, and microbiota which is frequently exposed to antacids and antibiotics. Resection of the ileocecal valve, in particular, appears to be a common finding in children with intestinal failure and SIBO, strongly suggesting the role of this structure in SIBO prevention. In line with this, Roland et al. (96) investigated the ileocecal junction pressure by WMC and the presence of SIBO by the LHBT and small bowel aspirate cultures. Interestingly, the authors found a combination of SIBO-predisposing factors in their patient cohort: (1) the small bowel transit time in SIBO-patients was significantly slower that those without SIBO; (2) the gastric pH was significantly higher in SIBO-patients than those without SIBO; and (3) the mean ileocecal junction pressure was significantly lower among SIBO-patients than those without SIBO. Thus, these findings reinforce the fact that an "incompetent" ileocecal valve predisposes to SIBO and emphasise the multifactorial nature of the disease.

Impoverished Conditions and Poor Socioeconomic Status

Impoverished conditions, which are commonly associated with a lack of basic sanitation services such as clean water, appropriate sewage, and collection of household garbage, may be a risk factor for SIBO. To our knowledge, six studies [one from Myanmar (21), three from Brazil (22–24), and two from Bangladesh (25, 26)] have investigated this association in the paediatric population.

Pereira et al. (21) investigated the prevalence of SIBO in children from a Burmese village (Myanmar) by using the LHBT. Around 85% of the village's population obtained drinking water from surface wells and ponds, and approximately 10% used rain water for the same purposes (97). The authors diagnosed SIBO in 27% of cases (53/195), with males being more commonly affected than females. Moreover, dos Reis et al. (22) found a significantly higher SIBO prevalence in children living in a slum as compared with controls living in households with appropriate sanitation services (37.5 vs. 2.1%, respectively). In addition, Mello et al. (23) also found a significantly higher SIBO prevalence in the slum-group compared with their socioeconomically advantaged counterparts (30.9 vs. 2.4%, respectively); however, it is important to note that the authors did not find statistically significant differences in the environmental variables (i.e., water contamination with coliforms, access to public water network, access to public sewage, and public collection of household garbage) between the SIBO-positive and SIBO-negative slum subgroups. The second and more recent study by Mello et al. (24) assessed SIBO prevalence and analysed the faecal gut microbiota of children residing in a Brazilian slum. Roughly 60% of children were found to have SIBO, and their faecal gut microbiota analyses showed a significantly higher number of *Salmonella* spp. and lower numbers of Firmicutes. Donowitz et al. (25), who investigated SIBO prevalence in 90 Bangladeshi children belonging to the lowest socioeconomic strata, reported that 16.7% had a positive GHBT and that the odds of developing SIBO were increased by the presence of an open drain/sewer outside the home (odds ratio, 4.78; 95% confidence interval, 1.06 to 21.62). More recently, Gaffar et al. (26) conducted a prospective nutritional intervention study on stunted and at-risk-of-stunting Bangladeshi children living in an urban slum (98), and found SIBO in 14.9% of subjects.

Taken together, these findings demonstrate that poor sanitation conditions, particularly contaminated water exposure, may expose children to a higher risk of developing SIBO. However, none of these studies provided a clear pathophysiological mechanism by which such unsanitary conditions predispose children to the disease. Donowitz et al. (55) proposed a mechanism of SIBO development in the setting of poor sanitary living conditions. The authors hypothesised that repeated exposure to abnormal levels of liposaccharides found in soil and drinking water may disrupt the MMC, causing faecal stasis (as seen in patients with gut dysmotility), thereby leading to SIBO development. While this theory appears to have some biological plausibility, to date, no studies have evaluated it.

Other SIBO Risk Factors

Immunodeficiency and coeliac disease (CD) have also been regarded as potential risk factors for SIBO development. As for immunodeficiency, we only identified one small-scale study (44) from 1990, which was conducted on children with immunodeficiency syndromes (i.e., IgA deficiency, hypogammaglobulinemia, and T cell defects). The most common clinical manifestation was chronic diarrhoea and SIBO was diagnosed in 41% of patients via jejunal aspirate culture. Moreover, in a more recent study (99), 4% ($n = 12/296$) of young adults with chronic diarrhoea and malabsorption syndrome were found to have hypogammaglobulinemia. Of these, 25% ($n = 3/12$) were diagnosed with SIBO. Although these findings show a relatively high SIBO prevalence in children and young adults with immunodeficiency syndromes, recent, large-scale studies are needed in order to support this association. As for CD, a recent systematic review (100) of adult patient studies found a high SIBO prevalence in patients with CD (20% pooled mean prevalence). The authors concluded that SIBO may be more common in patients with CD when symptoms do not improve after a gluten-free diet. We identified only two studies (101, 102) that characterized the microbiota composition and diversity of children with CD: subjects with active CD were found to have a higher abundance of members of the phylum Proteobacteria (101) and lower ecological indexes of genus *Lactobacillus* (102) as compared with healthy and non-active CD patients. Although these studies did not set out to evaluate SIBO *per se* and as such did not report CFU/g, the higher Proteobacteria abundance and lower ecological indexes of genus *Lactobacillus* seen in children with CD may indicate a disturbed microbial ecosystem. Certainly, a study that evaluates the presence of SIBO in coeliac children, either by the H_2/CH_4 breath test or small intestinal aspirate culture, would be of great interest.

It is important to note, however, that SIBO can develop even in the absence of any of the aforementioned risk factors. In line with the findings by Boissieu et al. (3) and our own experience, many children who test positive for SIBO are "healthy" and have no evident risk factors. There are clearly many questions to be addressed by future studies regarding the risk factors for SIBO in children.

AETIOLOGY OF PAEDIATRIC AND ADULT SIBO

SIBO can be caused by archaea or bacteria, by one or more microorganisms, by Gram-positive or Gram-negative bacteria, and by anaerobic or aerobic microorganisms (31). Pistiki et al. (103) conducted a cross-sectional study in which patients underwent duodenal aspirate cultures for the diagnosis of SIBO with a diagnostic threshold of $>10^3$ CFU/mL. SIBO was diagnosed in 20% of cases, being caused by one microorganism in 54.7% of cases and by two microorganisms in the remainder. In addition, the vast majority of bacterial isolates belonged to the phyla Proteobacteria and Firmicutes, with the most common being *Escherichia coli*, *Enterobacter* spp., *Klebsiella* spp., *Pseudomonas aeruginosa, Staphylococcus aureus, Acinetobacter*

baumannii, Stenotrophomonas maltophilia, Citrobacter freundii, Serratia marcescens, and *Enterococcus faecium* (in descending order of frequency). In turn, Pyleris et al. (104) conducted a study with the same diagnostic methodology, finding SIBO in 19.4% of adult subjects. Likewise, most bacterial isolates were members of the Proteobacteria and Firmicutes phyla (*Escherichia coli*, *Enterococcus* spp. *Klebsiella pneumoniae, Proteus mirabilis, Acinetobacter baumannii, Citrobacter freundii,* and *Serratia marscecens*), and in 75% of cases the overgrowth was due to one microorganism. In the remainder of cases, SIBO was caused by two microorganisms, with the most common dyads being *E. coli* + *K. pneumoniae* and *E. coli* + *Enterococcus* spp. Furthermore, Gutierrez (17) conducted a retrospective study on children with intestinal failure who underwent duodenal aspirate cultures. SIBO was diagnosed in 70% of cases by using a more stringent diagnostic threshold of $>10^5$ CFU/mL. Again, the most common causative microorganisms were members of the Proteobacteria and Firmicutes phyla, with the most common being *E. coli, Streptococcus viridans, K pneumoniae, Enterococcus* spp., and *Pseudomonas aeruginosa*. The overgrowth was due to more than one bacterium in a minority of patients. Moreover, Galloway (18) diagnosed SIBO in 43% of children with intestinal failure, of whom five out of six patients had overgrowth due to two different microorganisms and only one had overgrowth caused by one microorganism, with *Enterococcus* and *Klebsiella* being the most frequently isolated bacteria.

More recently, Ghoshal et al. (105) conducted a cross-sectional study on adult subjects with non-alcoholic steatohepatitis (NASH) who underwent a GHBT coupled with jejunal aspirate cultures. The authors defined SIBO as a bacterial growth of $\geq 10^5$ CFU/mL and low-grade bacterial overgrowth as $\geq 10^3$ CFU/mL; 20 and 60% of cases were diagnosed with SIBO and low-grade bacterial overgrowth, respectively. Amongst the SIBO cases, Proteobacteria (*Pseudomonas aeruginosa, Klebsiella pneumonia,* and *Acinetobacter* spp.) were the most commonly isolated microorganisms followed by members of the Firmicutes phylum (*Streptococcus* spp. and *Enterococcus faecalis*); 20% of cases were due to overgrowth of more than one microorganism.

Taken together, these findings demonstrate that in most cases, SIBO is caused by a single microorganism that belongs to the Proteobacteria phylum (37), particularly coliform bacteria such as *E. coli* and *Klebsiella spp*. However, it is important to consider three aspects: (1) culture-dependant approaches were used in these studies, which means that there was a risk of missing bacteria that remain difficult to culture under clinical laboratory conditions; (2) only two studies were conducted on children (17), and (3) SIBO was studied in a wide spectrum of clinical contexts, such as IBS, intestinal failure, and non-alcoholic steatohepatitis. Thus, the specificity of such microbiota alterations for SIBO must be interpreted in the context of these potentially important confounders.

Next generation sequencing (NGS) methods such as 16S ribosomal RNA (rRNA) sequencing have become a high-resolution and relatively cost-effective way to study the human gut microbiome (106–108). To our knowledge, only one study in children (6) has used these methods to evaluate the gut microbiota in the context of SIBO. Vonaesch et al. (6) conducted a novel, cross-sectional study on stunted and healthy children from Madagascar and Central African Republic (CAR), in which 16S rRNA amplicon sequencing was used to analyse the faecal gut microbiota composition of both groups and to confirm the presence of bacteria identified by gastric and duodenal fluid aspirate cultures—these were only obtained from the stunted group due to ethical reasons. The authors used the higher diagnostic threshold for SIBO diagnosis (i.e., $>10^5$ CFU/mL). Despite this, SIBO was diagnosed in 96% of stunted children (Madagascar: 100%; and CAR: 88%) and, interestingly, the most common causative microorganisms were oropharyngeal colonizers, such as *Streptococcus* spp., *Staphylococcus* spp., *Haemophilus* spp., *Moraxella* spp., and *Neisseria* spp. In addition, microbiota sequencing showed overrepresentation of oropharyngeal species (e.g., *Streptococcus* spp., *Haemophilus* spp., *Neisseria* spp., *Rothia* spp., *Actinomyces* spp., and *Gemella* spp.) and enteropathogens (e.g., *Escherichia coli, Shigella,* and *Campylobacter*), as well as underrepresentation of butyrate producers (e.g., *Clostridia* spp.) in stunted children compared with controls. In contrast with the studies described above (17, 103–105), however, most bacterial overgrowths were caused by members of the Firmicutes phylum followed by the Proteobacteria phylum.

CLINICAL FEATURES AND COMPLICATIONS OF SIBO IN CHILDREN

Paediatric SIBO is a heterogenous disorder that manifests itself through non-specific symptomatology, including gastrointestinal and non-gastrointestinal symptoms (31, 109). The most common signs and symptoms reported in the literature are chronic abdominal pain, abdominal distention, diarrhoea, flatulence, belching, steatorrhea, fetid stools, mucus in stools, fatigue, nausea, and stunted growth (**Table 1**) (2–6).

SIBO and Functional Gastrointestinal Disorders

It is always challenging to discuss functional gastrointestinal disorders (FGIDs) and their possible interaction with an organic aetiology, such as SIBO. A small number of observational studies have evaluated the association between FGIDs and SIBO in the paediatric population.

Korterink et al. (4) determined the presence of SIBO in children with abdominal pain–related functional gastrointestinal disorders [i.e., irritable bowel syndrome (IBS), functional abdominal pain (FAP), functional dyspepsia (FD), and FAP syndrome] by using the GHBT. Amongst the 14.3% SIBO-positive subjects, the most common symptoms were fatigue (75%), altered defecation pattern (71%), nausea (68%), and bloating (66%). However, only altered defecation pattern, loss of appetite, and belching were significantly higher than SIBO-negative subjects; diarrhoea and flatulence did not reach statistical significance. Furthermore, Collins et al. (46) also used the hydrogen/methane breath test to diagnose SIBO in their cohort of children with chronic abdominal pain (i.e., FD, IBS, and FAP). Ninety one percent of cases and 35% of healthy controls

had a positive breath test, respectively. Surprisingly, there were no differences in the presence of gastrointestinal symptoms such as bloating, gas, incomplete evacuation, constipation, diarrhoea, mucous in stool, or straining, between SIBO-positive and SIBO-negative subjects; however, it is important to mention that the comparison was disproportional between the two groups (68 vs. 7 patients, respectively). Moreover, Scarpellini et al. (45) evaluated the prevalence of SIBO in children with IBS by the lactulose hydrogen/methane breath test, finding a higher prevalence of SIBO amongst IBS sufferers compared to their healthy counterparts (65 vs. 7%, respectively). Taken together, these findings demonstrate that SIBO is a frequent underlying diagnosis in children with functional abdominal pain disorders (i.e., IBS and FD), thus suggesting a role in their pathogenesis.

The IBS-SIBO interaction has attracted a tremendous amount of research in the last decade, and thus it deserves special attention. A recent systematic review and metanalysis (110) of observational studies in adults estimated the prevalence and determined predictors of SIBO in IBS. The authors found an overall pooled SIBO prevalence of 38% (95% CI 32–44) as well as a 4.7 (95% CI 3.1–7.2) pooled OR of SIBO in IBS subjects as compared with healthy controls. Surprisingly, PPI use was not associated with SIBO. Even though a growing body of literature shows a clear association between the two disorders, it is unclear whether SIBO precedes IBS or vice versa (111, 112). Thus, until better data are available, children presenting with IBS-like symptomatology may merit SIBO-diagnostic workup, such as a H_2/CH_4 breath test [SIBO's role in IBS is comprehensively reviewed elsewhere (61, 113)].

Constipation, another FGID, has been associated with intestinal methane production in adults and children (methanogenic SIBO) (114) Indeed, a 2011 metanalysis (115) of nine studies (1,277 subjects) found a significant association between methane on breath test and constipation (OR 3.51, CI 2.00–6.16). Moreover, Pimentel et al. (116) demonstrated this association in animal and human models: intraluminal infusion of methane reduced small bowel transit by 59% in canine models as compared with controls, and methane was also found to increase intestinal contractile activity in guinea pigs and in patients with IBS. In contrast, Mello et al. (23) did not find an association between methane and constipation in their patient cohort of children living in a slum; in fact, none of these children had constipation despite a 30% prevalence of methanogenic SIBO. More recently, Ghoshal et al. (117) investigated the effect of rifaximin on breath methane and colonic transit in adult patients with constipation. The authors found that a larger percentage of patients with chronic constipation were methane producers (>10 ppm) and had slower colonic transit times as compared with controls. Methane producers ($n = 13$) were randomly assigned into two groups: rifaximin group (14-day trial) and placebo group. After treatment, the rifaximin group had a significantly lower area under the curve for methane production compared with the placebo group, and colonic transit time normalized in 66% of cases as compared with the placebo group, in whom colonic transit time never normalized. Thus, these findings support the association between methanogenic SIBO and constipation. The most important question to be answered is whether it would be cost-effective to perform methane breath tests on all children with "functional" constipation, considering the relatively high prevalence of the disease. What we know from small scale studies is that children with constipation and retentive faecal incontinence are more likely to be methane producers than children with constipation alone (15, 20). Thus, possibly, these children may benefit from antibiotic therapy. However, large scale, prospective studies are warranted in order to (1) clarify the relation between methanogenic SIBO and constipation, and (2) determine whether children with methanogenic SIBO and constipation may benefit from antibiotic treatment.

SIBO and Systemic Disorders

Increasing evidence suggests that SIBO may be implicated in the complex pathophysiology of stunted growth (6, 25) and environmental enteric dysfunction (EED; formerly known as environmental enteropathy) (24, 55, 56, 60). Stunting affects >30% of children under five from low income countries (118). As shown in **Figure 3**, intraluminal competition for micro and macronutrients between the excessive bacterial biomass and the host (119), as well as other SIBO-induced factors (e.g., diarrhoea, carbohydrate malabsorption, protein loss, increased intestinal permeability, intestinal and systemic inflammation), can lead to a negative caloric balance in the host, thereby, resulting in stunted growth and malnutrition. Such factors, too, characterise EED, thus it appears that SIBO may play an important role in EED's pathogenesis (6, 120). Vonaesch et al. (6) diagnosed SIBO in 96% of their patient cohort of children with stunting by the GHBT. In addition, Donowitz et al. (25) found a 16.7% SIBO prevalence in Bangladeshi children using the same diagnostic methodology. Taken together, these findings suggest a possible role of SIBO in stunting and EED's pathogenesis. Thus, it may be worthwhile to perform a hydrogen/methane breath test as part of the clinical approach to children with these diseases.

Moreover, although deficiencies of liposoluble vitamins (A, D, and E) and vitamin B 12 haven been documented in the adult population (121, 122), no studies have explored this issue in children with SIBO—a study assessing vitamin B12 and liposoluble vitamin status in children affected by SIBO would be of great interest. Menaquinone (vitamin K2) is produced by the gut microbiota (123), and thus from a physiological standpoint it would be contradictory to assume that vitamin K deficiency would arise in a bacterial-abundant environment. However, a recent case report described a 17-year-old female with vitamin K deficiency possibly caused by SIBO. The authors speculated that the vitamin K deficiency seen in this patient may have been the result of reduced menaquinone-producing bacteria, expansion of vitamin K-consuming bacteria, or severe malabsorption (124). Further gut metabolome studies (125) are needed to elucidate SIBO's role in vitamin deficiencies.

Currently, there are no guidelines regarding diagnosis or treatment of SIBO in children. Given the heterogenous and non-specific nature of the disease, it is sometimes challenging to decide in whom to initiate SIBO diagnostic work-up. However, it is of paramount importance to first rule out signs and symptoms (i.e., red flags) that may indicate diseases other than SIBO.

DIAGNOSIS

SIBO can be diagnosed by invasive and non-invasive methods. The non-invasive methods include breath tests, while invasive methods comprise culture-dependant and culture-independent approaches.

Hydrogen and Methane Breath Testing

Although subject to debate and controversy (126–128), the H_2 and CH_4 breath tests are increasingly being used due to their widely availability in healthcare facilities, because they are inexpensive, practical, and non-invasive (which is extremely important in paediatrics), and because the results can be interpreted on the same day of the test [the H_2/CH_4 breath test procedure is thoroughly reviewed elsewhere (92, 129, 130)]. According to the 2017 "Hydrogen and Methane-Based Breath Testing in Gastrointestinal Disorders: The North American Consensus"(92), a panel of 17 experts from North America and the latest consensus in regard to this topic, SIBO diagnosis is *suggested* when there is an increase in H_2 of $\geq$20 ppm over baseline within the first 90 min of the test with either lactulose or glucose, or when there is an increase in CH_4 of $\geq$10 ppm at any time point of the test (**Figure 4**). On the other hand, the older Rome Consensus (130) recommends the use of glucose as substrate due to its greater accuracy, and defines SIBO as an increase in H_2 of $\geq$12 above baseline by using the GHBT. A definition of SIBO by using the LHBT was not included in this paper.

In order to understand the mechanisms behind H_2 and CH_4 breath testing, one must be aware of two concepts: orocaecal transit time and intraluminal gas production. The human body has no means of producing H_2 and CH_4 other than intraluminal microbial fermentation and methanogenesis, respectively (92, 130–132). As previously mentioned, the colonic microbiota represents virtually the whole gut microbiota, most of which is composed of fibre-fermenter anaerobes, mainly those belonging to the Actinobacteria and Firmicutes phyla (133). Undigestible carbohydrates reaching the colon are readily fermented by hydrogenogens (H_2-producing bacteria) (134), and hence a physiological increase in H_2 is expected in the colon. Intraluminal H_2, in turn, is absorbed into the systemic circulation and is transported to the lungs where it can then be released through exhaled breath (129). Moreover, the orocaecal transit time—the elapsed time between ingestion of a substance until it reaches the caecum—has been shown to be around 90 min in both adults and children, as assessed by the LHBT (135). Thus, in the presence of SIBO, substrate administration will result in a small bowel H_2 peak, occurring before the orocaecal transit time has completed (i.e., 90 min).

Glucose and lactulose are the substrates usually used for breath tests to diagnose SIBO. The former is a monosaccharide which is readily absorbed in the proximal small intestine, and the latter is a synthetic, undigestible disaccharide that reaches the caecum intact. In the presence of SIBO, glucose administration will result in a H_2 peak which is produced in the small intestine (i.e., <90 min); in addition to this peak, lactulose administration can give rise to a second –colonic– H_2 peak: the early peak produced by the bacterial overgrowth in the small intestine and the second one caused by the colonic microbiota fermentation (92, 129). However, both substrates have their own advantages and limitations. By using glucose, false negative results can arise in the presence of bacterial overgrowth in the distal small bowel (i.e., ileum), as glucose is readily absorbed proximally. In addition, an increased orocecal transit time can give rise to false positive results as the substrate reaches the colon rapidly, thereby undergoing premature fermentation by colon bacteria—this can occur with both glucose and lactulose (126). False negative results can also arise in the absence of H_2-producing bacteria or in the presence of CH4-producing microorganisms (discussed below) (130). Based on a 2008 systematic review (136), the sensitivity and specificity of the GHBT ranged from 20 to 93% and 30 to 86%, respectively; and the sensitivity and specificity of the LHBT ranged from 31 to 68% and 44 to 100%, respectively.

Methanobrevibacter smithii, a member of the domain Archaea and the most abundant methanogen in the human alimentary tract produces CH_4 as an end product of hydrogen metabolism (hydrogenotrophic methanogenesis), by using one molecule of carbon dioxide and four molecules of H_2. The archaeon is extremely oxygen sensitive and relies completely on hydrogenogens for CH_4 production, due to its inability to metabolise monosaccharides. For this reason, *Methanobrevibacter smithii* is considered an "obligate cross-feeder" (131). Furthermore, since the archaeon utilizes H_2 to produce CH4, it can lead to a falsely negative HBT. Thus, it is recommended that both H_2 and CH4 are measured in order to avoid this (92).

In conclusion, despite the limitations of the H_2/CH_4 test (128), it remains a useful tool in the diagnosis of paediatric SIBO due to its practical and non-invasive nature. Importantly, there are no reported significant side effects for the hydrogen/methane breath test other than transient abdominal pain or vomiting during the test.

Culture-Dependant Approaches

Culture-dependent approaches are considered the current gold standard for definitive SIBO diagnosis. A small intestinal (i.e., duodenum or jejunum) aspirate is obtained at upper gastrointestinal endoscopy through a sterile catheter and stored anaerobically prior to culture for both aerobic and anaerobic bacteria. When using the proximal jejunal aspirate culture, a bacterial concentration of $>10^3$ CFU/mL is regarded as indicative of SIBO (92, 109), although there is some heterogeneity in the literature in this regard and some experts recommend a threshold of $>10^5$ CFU/mL as more specific (137). Despite being regarded as the gold standard diagnostic method, there are several notable limitations to this approach. Firstly, endoscopy-guided aspiration procedures are invasive, expensive, and require specialist input, limiting its availability and application in certain settings. Secondly, we know from microbiome research that standard clinical laboratory culture-based approaches have the potential to detect only a minority of the extant bacterial consortium. In addition, care must also be taken not to contaminate samples or sampling apparatus with oropharyngeal microbes, thereby contributing to false positives. Likewise,

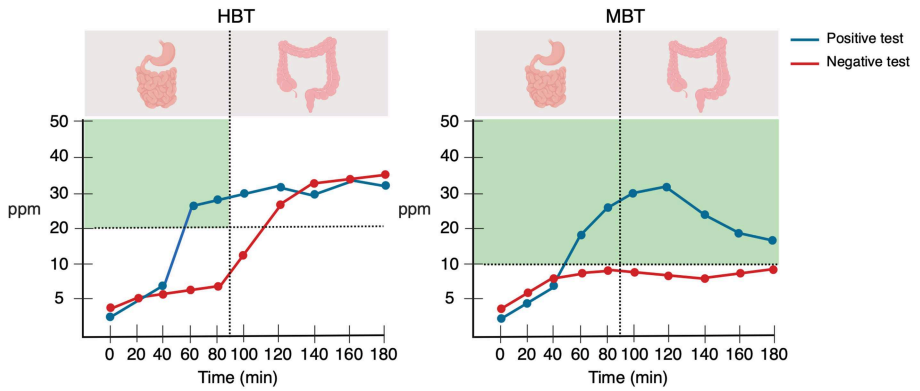

FIGURE 4 | Graphical representation of the hydrogen and methane breath tests. The vertical dotted line indicates the completion of the orocecal transit time, and the horizontal dotted line indicates the current diagnostic thresholds for SIBO. The green shaded areas indicate where the test is considered positive. Created with BioRender.com.

the significant heterogeneity in aspirate sampling protocols are known to impact substantially on the test outcome and accuracy. For example, one study previously used air, rather than nitrogen or carbon dioxide, during endoscopy to recover aspirate samples in suspected SIBO and cultured anaerobic bacteria from just one of 50 samples (138). Other variables include specific site of sampling and volume of aspirate. The lack of standardised protocol and diagnostic consensus for the gold standard investigation undoubtedly contributes to the massive variation in the SIBO prevalence and incidence rates reported in the literature. This, in turn, limits comparability between studies and ultimately hinders research progression in the field. In order for the development of a greater understanding and novel therapies for the disease to arise, the disparities in protocols and diagnostic criteria should first be addressed at a global scale. Finally, in the context of paediatric SIBO, endoscopy of seemingly healthy control children appears to present an obstacle in clinical research studies due to ethical considerations and, therefore, development of less invasive diagnostic approaches is warranted.

SIBO in the Era of Next Generation Sequencing

As eluded to previously, culture-independent approaches based on NGS technologies have become the most widely applied modalities for studying both the composition and metabolic activity of the gut microbiota. There are several considerations to be addressed when selecting a methodology, including sample type, DNA extraction method, sequencing modality and platform, as well as bioinformatic pipeline [reviewed comprehensively in (43)].

In general, the composition of the gut microbiota is currently most cost effectively studied through 16S rRNA sequencing. The 16S region is a highly conserved region of rRNA within all taxa of bacteria, which displays sufficient variation and divergence in parts to allow differentiation at the genus level. Although it has been central to the progression of the field of microbiome research, 16S rRNA sequencing is crucially limited in the fact that it does not generate absolute data on quantities of bacteria, but rather provides investigators with a relative abundance of each taxa within a sample. The second modality which is indeed worthy of consideration is metagenomic shotgun sequencing. While 16S rRNA sequencing provides an overview of microbiota composition, metagenomic shotgun sequencing goes a step further by telling us who is there and what are they capable of in metabolic terms.

Although such sequencing capabilities are now commonplace in microbiological and medical research laboratories the globe over, uptake and application to SIBO research has been comparatively slow and, as a result, there is a dearth of relevant clinical data available. Despite this, several clinical studies which targeted alternate, but related, gastrointestinal disorders have provided some intriguing data on the disorder. In line with this, some small studies have reported on the gut microbiome of IBS cohorts, many of which were confirmed to suffer from concomitant SIBO. One such study investigated the faecal microbiota of a cohort of 30 Chinese patients with diarrhoea-predominant IBS, 14 of which were also confirmed to have SIBO by LHBT (139). At baseline, this cohort displayed reduced microbiota diversity, increased relative abundances of *Bacteroidetes* and decreased relative abundances of *Firmicutes* when compared to the healthy controls. In addition, the IBS cohort was shown to have reduced relative abundances of the genus *Lactobacillus*, as well as several genera associated with butyrate production. The investigators subsequently implemented a 2-week course of rifaximin, after which clinical symptoms improved and repeat LHBT demonstrated remission in 65% of the SIBO-positive subjects. However, the microbiota post-treatment displayed only minor alterations in taxa for which little metabolic information is available, a result which was mirrored previously in similarly designed trials of rifaximin in IBS (140, 141). Indeed, it must be reiterated that less than half of the IBD cohort studied in this trial were confirmed to have concomitant SIBO and the samples investigated (i.e., faeces) bears little resemblance to the site most relevant to the disease; therefore, no definitive conclusions on SIBO pathogenesis should be drawn from this data.

With regards to SIBO in children, one study outlined above aimed to investigate the microbiota in sub-Saharan children with stunted growth (6). In addition to faecal microbiota analysis, it was deemed pertinent to retrieve and analyse the microbiota of small intestinal aspirates, due to its role in nutrient absorption and malnutrition. The authors declared a form of microbial "decompartmentalization," in which oropharyngeal-associated microbes were found to be over-represented in the small intestine of these children, 91% of which tested positive for SIBO. Although this was deduced primarily from culture-based methods, it is consistent with preliminary reports suggesting that SIBO is not caused by migratory colonic microbes (142). In turn, 16S rRNA sequencing of the duodenal microbiota in these subjects revealed a community containing near-equal parts *Proteobacteria* (32.4%), *Bacteroidetes* (29.6%), and *Firmicutes* (25.6%), with lesser portions of *Fusobacteria* (9.2%), and *Actinobacteria* (1.7%). Critically, however, this study was severely limited by the fact that there are no appropriate controls available for the microbiota analysis of duodenal aspirates, presumably due to ethical considerations. Therefore, we are left, once again, with half-truths and a degree of speculation on the potential role and composition of the small intestinal microbiota in SIBO.

These studies demonstrate that SIBO has been regarded largely as a sign or sequela of a gastrointestinal disorder, rather than a discrete disorder in itself. This perspective has meant that the pathogenesis of SIBO has sparsely been addressed directly, but rather has been of peripheral interest in studies with an intersecting interest. Having said this, perhaps the most informative characterisation effort of SIBO to date has come from a recent investigation of 126 adults displaying gastrointestinal symptoms (66 SIBO positive and 60 SIBO negative) (143). The authors uncovered that, although duodenal aspirate culture results do not correlate with symptoms, the aspirate microbiome was significantly altered in symptomatic participants. This altered microbiome is characterised primarily by decreased levels of the genus *Prevotella* and enhanced microbial metabolism of ascorbic acid. In addition, it was identified that age, recent antibiotic exposure, PPI use and diet were the major proponents in the disruption of the microbiome and onset of symptoms. In line with this, the investigators demonstrated that a dietary fibre restriction intervention in healthy high-fibre consuming individuals had significant effects on the microbiome and triggered gastrointestinal symptoms common to SIBO. This study identifies several potentially targetable components of SIBO pathogenesis and represents an excellent blueprint for the future study of the disease.

TREATMENT

Due to the relative inaccessibility of duodenal samples for culture and difficulty in differentiating the culpable microorganism in a diverse ecosystem such as the small intestine, antibiotic therapy is generally initiated on an empiric basis. A 2013 systematic review and meta-analysis of antibiotic use in the context of SIBO found that rifaximin was by far the most commonly applied (144). While the meta-analysis ruled in favour of antibiotic use over placebo (effectiveness ratio 2.55, CI 1.29–5.04), rifaximin failed to reach a significant superiority (effectiveness ratio 1.97, CI 0.93–4.17). However, just three studies were deemed appropriate for this analysis and their heterogeneity limit the usefulness of this result. In line with this, the systematic review went on to reveal that monotherapy with 1,200 mg/d rifaximin was efficacious, at 60.8% remission. Moreover, the antibiotic reached a substantially heightened efficacy of 85% when combined with partially hydrolysed guar gum, albeit in a single trial. Two studies included in the systematic review investigated the use of metronidazole, demonstrating a return to normal breath test in 51% of SIBO patients; while a single small study reported remission in all 14 patients recruited and treated with ciprofloxacin.

A more recent systematic review and meta-analysis of rifaximin therapy in SIBO included data from >1,300 patients (145). A dose-dependent response was demonstrated for eradication rates and, in line with the previous systematic review, the most commonly used dose was 1,200 mg/d, with one study reporting 600 mg/d and another 1,600 mg/d. The investigators found an overall eradication rate of 70%, with adverse events reported in < 5%. In addition, in a subset of studies which assessed symptom severity and resolution, meta-analysis revealed remission from symptomology in 68% of patients who were found to be successfully eradicated of SIBO. These studies demonstrate the efficacy and safety of rifaximin in the treatment of SIBO and its symptoms.

While the studies included in the above meta-analysis were all conducted with adult cohorts, one study previously investigated the use of rifaximin in children (48). Applying a regimen of 600 mg/d for 1 week, the investigators reported a rate of 64% (n = 21/33) in breath test normalisation response. In light of the limited available data, a dose response study of rifaximin in a paediatric population would indeed be of interest. Finally, one study investigated the efficacy of a combination regimen of trimethoprim-sulfamethoxazole (TMP-SMT; 30 mg kg^1 d^1) and metronidazole (MTZ; 20 mg kg^1 d^1) twice daily for 2 weeks in slum-dwelling children suffering from SIBO (146). When retested for SIBO by breath test 1 month after commencement of this therapy, the authors noted 95% (n = 19/20) resolution of the disorder. However, the lack of a placebo or non-intervention control group limits assessment of temporal effects on SIBO status. Taken together, these results indicate that antibiotics are an effective means of treating SIBO in children and, although non-rifaximin antibiotics appear to produce greater resolution, the data is weaker for such medications and rifaximin has demonstrated safety in a greater number of participants and studies.

While antibiotics remain the first-line and gold-standard approach to SIBO management, there are additional or alternative approaches that may have application in the future, but the efficacy of which remains uncertain at present. For instance, there is biologic plausibility to the hypothesis that there may be a role for low FODMAP (Fermentable Oligosaccharides, Disaccharides, Monosaccharides, and Polyols)

diets in decreasing fermentable substrates in the context of SIBO. Low FODMAP diets aim to greatly deplete or entirely eliminate the highly fermentable simple carbohydrates which are commonly found in certain dairy products, fruits, vegetables, nuts and legumes, with the ultimate aim of graded reintroduction of specific FODMAPs to elucidate the culpable source (147).

Interestingly, probiotics have also previously been considered as potential agents in the management of SIBO (148). In line with this, a more recent meta-analysis evaluating the efficacy of this avenue revealed that probiotic intervention resulted in vastly reduced H_2 levels (WMD −36.35, CI −44.23−28.47) and increased rates of decontamination (RR 3.29, CI 1.47–7.36) when compared to placebo. More intriguingly, however, is that probiotics also demonstrated more favourable results when compared directly to metronidazole treatment (RR 1.49, CI 1.07–2.08). Despite this, the best results overall were obtained when probiotics were combined with rifaximin or minocycline.

Finally, there is some evidence to suggest that certain statins may have a role in depleting *Methanobrevibacter* spp., thereby offering a novel therapy for methane-specific SIBO (149, 150). In line with this, a modified-release formulation of lovastatin, termed SYN-010, has been created to deliver the drug in a biphasic manner during transit, thereby avoiding considerable degradation and absorption in the upper gastrointestinal tract (151). A dose of 42 mg/day of SYN-010 for as little as seven days was demonstrated to significantly reduce methane production when compared to placebo in a multi-centred double-blind RCT (152). Moreover, this promising therapy recently entered an efficacy and safety trial in patients with IBS (ClinicalTrials.gov ID: NCT03763175). The effect of this drug appears to be primarily due to the HMG-CoA reductase inhibition attributes of statins, thereby preventing the formation of mevalonate, a primary precursor in of key membrane lipids specific to archaea. This mechanism, along with several others, has been previously reviewed extensively elsewhere (41), and so, will not be discussed further herein.

PROGNOSIS

At present, it is unclear whether the gut microbiota changes that characterise paediatric SIBO have detrimental effects in the long-term. In order to shed light on the long, and even short-term prognosis of children with SIBO, large scale, prospective studies are warranted.

CONCLUSION

Herein, we attempted to synthesise the most current literature on paediatric SIBO, from a gut microbiota perspective. Paediatric SIBO is a heterogenous and poorly understood entity, whose prevalence and incidence are difficult to determine due to the lack of uniformity and consensus of its diagnostic criteria. However, based upon the available literature, it appears that SIBO is a common underlying diagnosis in children who present clinically with certain FGDs and stunted growth, and in children with history of acid suppressive therapies, intestinal motility disturbances, anatomical alterations, or impoverished conditions. Further research with integration of culture-dependant and culture-independent approaches is needed in order to (1) understand paediatric SIBO pathogenesis, clinical presentation, and prognosis, and (2) establish global consensus on the diagnostic criteria for SIBO.

AUTHOR CONTRIBUTIONS

DA contributed conception and design of the manuscript. DA and PR wrote the first draft of the manuscript. ET, JR, and EQ wrote and edited sections of the manuscript. All the authors contributed to manuscript revision, read, and approved the submitted version.

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REVIEW
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Current Trends in Tolerance Induction in Cow's Milk Allergy: From Passive to Proactive Strategies

Cansin Sackesen[1†], Derya Ufuk Altintas[2†], Aysen Bingol[3†], Gulbin Bingol[4†], Betul Buyuktiryaki[5†], Esen Demir[6†], Aydan Kansu[7†], Zarife Kuloglu[7†], Zeynep Tamay[8†] and Bulent Enis Sekerel[5†]*

[1] Division of Pediatric Allergy, Department of Pediatrics, Koc University School of Medicine, Istanbul, Turkey, [2] Division of Pediatric Allergy, Department of Pediatrics, Cukurova University School of Medicine, Adana, Turkey, [3] Division of Pediatric Allergy and Immunology, Department of Pediatrics, Akdeniz University Faculty of Medicine, Antalya, Turkey, [4] Division of Pediatric Allergy and Immunology, Department of Pediatrics, Acibadem University School of Medicine, Istanbul, Turkey, [5] Division of Pediatric Allergy and Asthma, Department of Pediatrics, Hacettepe University Faculty of Medicine, Ankara, Turkey, [6] Division of Pediatric Allergy, Department of Pediatrics, Ege University School of Medicine, Izmir, Turkey, [7] Division of Pediatric Gastroenterology, Department of Pediatrics, Ankara University School of Medicine, Ankara, Turkey, [8] Division of Pediatric Allergy and Immunology, Department of Pediatrics, Istanbul University Istanbul School of Medicine, Istanbul, Turkey

This review addresses the current strategies of inducing tolerance development in infant and childhood cow's milk protein allergy (CMPA). The change in prevention strategies for CMPA has been emphasized based on the lack of evidence to support the efficacy of food allergen avoidance in infancy and the concept of the dual-allergen-exposure hypothesis, which suggests that allergen exposure through the skin leads to sensitization, whereas early oral consumption of allergenic food protein induces oral tolerance. The new approach is based on the likelihood of early introduction of allergenic foods to the infant's diet to reduce the development of food allergies through oral tolerance induction. The latest treatment guidelines recommend the continuation of breast feeding and the elimination of cow's milk and products from the maternal diet in exclusively breast-fed infants with CMPA, the use of an extensively hydrolyzed infant formula (eHF) with proven efficacy in CMPA as the first elimination diet in formula-fed infants with CMPA and the use of amino acid-based formula (AAF) in severe cases, such as anaphylaxis, enteropathy, eosinophilic esophagitis, and food protein-induced enterocolitis syndrome (FPIES), as well as cases of multiple system involvement, multiple food allergies, and intolerance to extensively hydrolyzed formula (eHF). In conclusion, this paper presents the current knowledge on tolerance development in infants and children with CMPA to increase the awareness of the clinicians concerning the new approaches in CMPA treatment Tolerance development is considered a relatively new concept in CMPA, inducing a shift in interventions in CMPA from a passive (avoidance of responsible allergen) toward a proactive (tolerance induction) strategy.

Keywords: cow's milk protein allergy, prevention, tolerance, formula, allergenic foods

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***Correspondence:**
Cansin Sackesen
csackesen@yahoo.com;
csackesen@ku.edu.tr

† These authors have contributed equally to this work

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INTRODUCTION

There has been an alteration in the natural history of food allergy during the previous two decades with an increased prevalence, more severe clinical manifestations and higher risk of persistence into later ages (1–4).

Given that oral exposure is considered to be responsible for allergic sensitization to food, an elimination diet has become the best strategy for the prevention of food allergies, and food allergen avoidance has been the mainstay preventive strategy in food allergy (4–7). However, in addition to the lack of convincing evidence to support the long-term efficacy of food allergen avoidance in the prevention of food allergy, the likelihood of the development of sensitization via allergen exposure through the skin and the induction of tolerance via early oral consumption of allergenic food proteins has also been suggested (4–6).

Accordingly, prior recommendations for the avoidance of peanuts during the first 3 years of life and common food allergens until the first (milk), second (egg), or third (tree nuts and fish) years of life in a diet of an infant at increased risk of atopy by the American Academy of Pediatrics guidelines (7) have been withdrawn and replaced by comments about the lack of current evidence on these topics (8).

Cow's milk protein allergy (CMPA), the most common food allergy in infants and young children (9–13), can induce a diverse range of symptoms involving many different organ systems depending on the type of immune reaction (14). The discrimination of the type of immune reactions is important, since patients with IgE-positive vs. IgE-negative CMPA are considered at increased risk of developing multiple food allergies and atopic diseases, such as asthma, atopic dermatitis, and rhino-conjunctivitis, as well as delayed tolerance (15–18).

Without an appropriate diagnostic workup, there is a risk for both over- and underdiagnosis of CMPA leading to inappropriate elimination diets and thus impaired growth and a poor quality of life (14, 19, 20).

Therefore, this review by experts aimed to provide a guiding document and a comprehensive framework for addressing tolerance development in infants and children with CMPA. The main topics addressed in this paper include (a) an overview of food allergy (prevalence and epidemiology, tolerance development, and risk factors for persistence), (b) CMPA (prevalence, types of immune reaction, clinical presentation, and associated conditions, natural course, and tolerance development), and (c) interventions for allergen avoidance and induction of tolerance (baked milk products, formulas, and immunotherapy) in patients with established CMPA.

Abbreviations: AAF, amino acid-based formula; CMP, Cow's Milk Protein; CMPA, Cow's Milk Protein Allergy; EAT, Enquiring about Tolerance Trial; eHF, extensively Hydrolyzed Formula; EoE, Eosinophilic esophagitis; FPIES, Food protein-induced enterocolitis syndrome; LEAP, Learning Early about Peanut Allergy; OFC, Oral Food Challenge; OIT, Oral Immunotherapy; RCT, Randomized Controlled Trials; sIgE, specific Immunoglobulin E; SPT, Skin Prick Test.

OVERVIEW OF FOOD ALLERGY

Prevalence and Epidemiology of Food Allergy

According to systematic reviews and meta-analyses of epidemiological studies during the last 2 decades, the frequency of food allergy appears to be increasing in both developed and developing countries, particularly in children (21–25). The prevalence of an oral food challenge (OFC)-proven food allergy is considered to range from 1 to 10% in infants and preschool children (<5 years) and from 0.16 to 2.5% in school-aged children (>5 years) (21).

Food allergy can be induced by IgE-mediated mechanisms, manifesting as immediate urticaria, vomiting, wheezing and anaphylaxis, non-IgE-mediated delayed cell-mediated reactions, or mixed immune reactions to any routes of exposure to culprit foods (3, 22, 26, 27).

The most prevalent food allergens in childhood are cow's milk and eggs, while the third and fourth most common triggers differ depending on the geographical region, age, and dietary patterns, such as peanuts in the United States and Switzerland, wheat in Germany and Japan, tree nuts in Spain, sesame in Israel, walnuts in Korea, and hazelnut in Turkey (3, 10, 28–30).

Food allergy may affect several organs, including the skin, gastrointestinal tract, respiratory tract, and cardiovascular system; moreover, food-induced anaphylaxis is considered to be the most serious and potentially life-threatening reaction, with the highest prevalence in the 0–4 year age group and an increased prevalence in the last two decades, particularly in the 5–14 year age group (3, 26, 27, 31, 32).

Tolerance Development and Risk Factors for Persistence of Food Allergy

Symptom severity following ingestion, lower reaction eliciting threshold dose, earlier age at diagnosis, and presence and severity of other allergic comorbidities (e.g., eczema, asthma, allergic rhinitis) have been associated with a delayed amelioration of allergy to foods and a higher likelihood of a more persistent food allergy phenotype (33).

Routinely available assays of IgE sensitization include the skin prick test (SPT) and serum food-specific (s) IgE levels. In general, a larger SPT wheal size or higher food sIgE levels are associated with persistent food allergy (33).

Genetic factors, such as atopic family history, male sex, parental ethnicity, atopic dermatitis and related genetic polymorphisms, are important in the development of food allergy (6, 34, 35). Nevertheless, a rapid increase in food allergy prevalence over a short period necessitated evolving strategies to promote allergy prevention through the identification of modifiable environmental factors (i.e., food exposure, the intrauterine environment, and lifestyle factors) (6, 34, 35).

Food allergen avoidance during infancy had limited success in reducing food sensitization and food allergy after the first year of life and has no effect on respiratory allergy or aeroallergen sensitization from birth to age 4 years (35, 36). Moreover, no

convincing evidence exists on the benefit of exclusive breast-feeding beyond 4 months of age in preventing atopic disease and in reducing long-term IgE-mediated food allergy in children (6, 35). A recent study showed that cow's milk allergy was less frequent when regular CMP formula was introduced from the first 15 days of life as a complement to breastfeeding than when introduction at an age of 4–6 months (37).

The Learning Early about Peanut Allergy (LEAP) trial showed that the early consumption of peanut compared with avoidance was associated with a significant reduction in the development of peanut allergy by 5 years of age both in high-risk peanut sensitized infants with severe eczema and/or egg allergy (10.6 vs. 35.3%) and in peanut non-sensitized infants (1.9 vs. 13.7%) (38). Thus, early environmental exposure (through the skin) to peanut is considered to account for early sensitization along with the potential contribution of early oral exposure in the development of immune tolerance (5, 38). The Persistence of Oral Tolerance to Peanut (LEAP-On) study showed that the absence of reactivity was maintained in these infants with early consumption of peanut (39).

In the Enquiring about Tolerance (EAT) trial, early introduction of common dietary allergens (peanut, cooked hen's egg, cow's milk, sesame, white fish, and wheat) in small amounts from 3 months of age was studied in exclusively breast-fed infants ($n = 1{,}303$) compared to infants who were exclusively breastfed until 6 month of age (40). The results showed that early introduction and regular consumption of 2 grams of peanut and egg white protein per week was significantly associated with a lower prevalence of peanut and egg allergy between 1 and 3 years of age compared to infants who were exclusively breastfed for ∼6 months (40). However, the early introduction and regular consumption of cow's milk, sesame, white fish, and wheat was not as successful as peanut and egg in this study. We can conclude that randomized controlled trials of oral tolerance induction with early introduction of a group of foods (peanut, egg, milk, sesame, fish, or wheat) obtained variable results. Regarding the limitations of these studies, the meta-analysis by Ierodiakonou et al. (41) reported that early introduction of egg or peanut to the infant diet resulted in a lower risk of developing egg or peanut allergy with moderate certainty; however, the findings for early introduction of milk or hydrolyzed formula were classified as no evidence (42).

Two possible explanations have been proposed for the failure of allergen avoidance in infancy to prevent food allergy. These explanations include the likelihood of sensitization to food allergens to occur through routes of exposure other than oral consumption and the likelihood of the early introduction of some allergenic foods to the infant's diet to reduce the development of food allergies through oral tolerance induction (5, 43).

In this regard, the current concept of the "dual-allergen-exposure hypothesis" suggests that early cutaneous exposure to food protein via disrupted skin barrier results in allergic sensitization, whereas early oral exposure to food allergen induces tolerance (4–6, 40). Low-dose cutaneous exposure to environmental foods (on hands, tabletops, and dust) is considered to penetrate the skin barrier and induce T helper (h) 2 responses and IgE production by B cells. However, early high-dose oral consumption induces tolerance via Th1 and regulatory T-cell responses in the gut-associated lymphoid tissue. The timing and balance of cutaneous and oral exposure are thought to determine whether a child will have an allergy or tolerance (5, 43).

Based on the dual antigen exposure hypothesis, it has been suggested that (a) prompt intensive treatment of eczema in early infancy may decrease inflammation and permeability in the skin and prevent allergic sensitization to foods, (b) reduction in food allergens in the child's environment may lead to a reduction in sensitization, and (c) early introduction of allergenic foods to the infant's diet in small amounts may reduce the development of food allergies through oral tolerance induction (5, 43).

COW'S MILK PROTEIN ALLERGY

Clinical Presentations According to Type of Immune Reaction in CMPA

CMPA is the most common food allergy in infants and young children with a prevalence of 2–7.5%, which accounts for approximately one-fifth of childhood food allergies (1, 9–12, 14, 33, 44).

CMPA is a complex disorder caused by an aberrant inflammatory immune reaction to CMP, classified as "immediate" (up to 2 h after allergen ingestion, typically IgE-mediated) or "late onset" (up to 48 h, typically non-IgE or mixed type) adverse reactions that are distinct from those related to cow's milk intolerances (i.e., lactose intolerance) (9–12, 14, 18, 28, 45–47). CMPA is more prevalent in infants (2–6%) than in adults (0.1– 0.5%), and the disease peaks in the first year of life with a predominance of the IgE-mediated type of allergy (14, 16, 45). According to data from the EuroPrevall birth cohort, the incidences of overall CMPA and IgE-mediated CMPA in the first 2 years of life were reported to be 0.54 and 0.44%, respectively, while the incidence of non-IgE-mediated confirmed CMPA was reported to range from 0.13 to 0.72% across Europe (16).

In most children with CMPA, IgE-mediated CMPA predominates as manifested by generalized systemic reactions (anaphylaxis) or cutaneous, gastrointestinal and/or respiratory reactions along with positive skin tests and/or serum milk sIgE antibodies (28). Disorders involving non-IgE-mediated CMPA only occur in a subset of children and are mainly localized to the gastrointestinal tract, while skin (atopic dermatitis) and rarely respiratory tract reactions (Heiner syndrome) may also occur along with negativity of tests for IgE antibodies (28).

Despite the likelihood of an overlap of clinical symptoms in IgE-mediated and non-IgE-mediated type immune reactions and combinations of immediate and delayed reactions to the same allergen in the same patient, a detailed history and appropriate laboratory studies indicate the correct diagnosis in most cases (14, 28, 48). Distinguishing between the mechanisms of immune reactions is important, as IgE-mediated CMPA is associated with a higher risk of multiple food allergies and atopic conditions, such as asthma, later in life (18, 28, 49).

Overall, the clinical manifestations of IgE-mediated CMPA comprise cutaneous symptoms (70–75%: urticaria,

generalized maculopapular rashes, flushing, and angioedema), gastrointestinal symptoms (13–34%: nausea, vomiting, colicky abdominal pain, and diarrhea), respiratory problems (1–8%: nasal pruritus and congestion, rhinorrhea, sneezing, wheezing, dyspnea, chest tightness, and symptoms of asthma and allergic rhinitis), alterations that affect more than one organ system (26%), and severe and potentially life-threatening anaphylaxis (1–4%) (**Table 1**) (17, 50).

The majority of disorders involving non-IgE-mediated CMPA are localized to the gastrointestinal tract (nausea, vomiting, diarrhea, abdominal pain, blood in stool malabsorption, and failure to thrive or weight loss), and in some cases, atopic dermatitis symptoms may present at the same time (**Table 1**) (28, 50).

Gastrointestinal symptoms of non-IgE mediated CMPA are characterized by subacute and/or chronic symptoms and may present as a variety of disorders, including cricopharyngeal spasm, gastro-esophageal reflux disease (GERD), allergic eosinophilic esophagitis (EoE), food protein-induced enterocolitis syndrome (FPIES), food protein-induced allergic proctocolitis (FPIAP), food protein-induced enteropathy (FPE), and cow's milk-induced iron deficiency anemia (**Table 1**) (28, 50, 51).

EoE has become more prevalent over the past decade and is characterized by dysphagia, chest and abdominal pain, food impaction and food refusal, and failure to thrive or weight loss in the more severe cases, which are unresponsive to anti-reflux medications (28).

CMPA is one of the most common causes of FPIES, a form of non-IgE-mediated allergy that develops 1–3 h after the ingestion of milk protein in the acute form and results in repetitive vomiting, hypotonia, pallor, and, in some cases, hypotension and diarrhea (28, 52). Chronic FPIES is an uncommon form that occurs in children with daily consumption of the offending food resulting in chronic or intermittent emesis or reflux, watery diarrhea, and weight loss or failure to thrive (52, 53). FPE is an uncommon disorder that typically presents as diarrhea, failure to thrive, vomiting, and occasionally hypoproteinemia. FPIAP is a relatively benign disorder that results in mild rectal bleeding (i.e., flecks of blood) that may be accompanied with mild diarrhea in an otherwise healthy infant (28).

Heiner's Syndrome is a very rare form of CMPA-related pulmonary hemosiderosis and characterized by recurrent pulmonary infiltrates associated with chronic cough, recurrent fevers, wheezing, rales, tachypnea, and failure to thrive (28).

Natural Course of CMPA and Development of Tolerance

CMPA has a favorable prognosis with a natural course of onset from the neonatal period, a peak during the first year of life, and remission, with the majority of patients outgrowing the allergy throughout childhood and early adolescence (28, 33).

The reported rates of milk allergy resolution vary by IgE status, genetics, selection criteria, assessment methods, frequency of re-challenge, and study design, while trends toward a delayed resolution of allergy in CMPA and an atopic carrier status in infants who initially recover from CMPA later in life have also been emphasized (1, 28, 33, 43, 54).

The mechanism of the immune reaction in CMPA was shown to be associated with both the rate and timing of tolerance development, with more frequent and earlier development of tolerance in non-IgE mediated CMPA than in IgE-mediated CMPA (16). However, a more favorable prognosis for IgE-mediated CMPA with 65–75% resolution rates until the age of 3–4 years has also been reported in population-based studies (55, 56). On the other hand, the tolerance acquisition in FPIES (non-IgE mediated food allergy) may be delayed when the patient has the co-existence of IgE sensitization to milk (57). Another large population-based cohort study from Israel reported that in patients with CM-induced FPIES, 60% had tolerance by 1 year, 75% by 2 years, and 85% by 3 years (58). Moreover, it is reported that the development of tolerance in cow's milk-induced FPIES occurs earlier than grain-induced and solid food (e.g., egg)-induced FPIES (52).

The levels of sIgE (particularly against sequential epitopes of casein) and antibody binding to other ingestant and inhalant allergens, SPT wheal sizes, severity of eczema at diagnosis, respiratory symptoms with skin and/or gastrointestinal symptoms at onset, persistence of serious symptoms, sensitization to multiple foods, initial sensitization to respiratory allergens and family history of progression to atopic asthma, rhinitis, and eczema were reported to be inversely associated with the timing of CMPA resolution, leading to a higher risk of a longer duration of disease (17, 28, 33, 59). A larger SPT wheal size and/or higher food-sIgE levels at the initial diagnosis were associated with a higher likelihood of persistent food allergy (33). Furthermore, patients with milk sIgE-positive vs. milk sIgE-negative CMPA are at increased risk of developing multiple food allergies as well as atopic diseases, such as asthma, atopic dermatitis, and rhino-conjunctivitis (15).

INTERVENTION STRATEGIES FOR TOLERANCE INDUCTION IN CMPA

Overall, the intervention strategies in CMPA have been targeted at three levels: (1) primary prevention of initial IgE sensitization; (2) secondary prevention of the triggering of allergic reactions to interrupt the development of food allergy in IgE-sensitized children; and (3) tertiary prevention to reduce the manifestation of end-organ allergic disease in children with established food allergy via avoidance of allergenic food and induction of tolerance (i.e., baked milk products, formulas, and oral immunotherapy) (6, 45).

For at-risk infants unable to be exclusively breast-fed, the use of hypoallergenic hydrolyzed formulas during the critical risk period for allergic sensitization has been suggested as a preventive strategy (6, 45). However, no evidence exists to support feeding with a hydrolyzed formula for the prevention of allergy compared with exclusive breast-feeding or cow's milk formula (6, 41, 42, 60). No evidence exists either regarding the potential inhibitory role of implementation of an elimination diet or use of supplements (i.e., probiotics) during pregnancy or lactation in development

TABLE 1 | Clinical presentation of CMPA with respect to type of immune reaction (14, 18, 28).

Presenting symptoms/signs	**IgE-mediated CMPA**		**Non-IgE-mediated CMPA**	
Gastrointestinal	Nausea/vomiting Diarrhea Colic Constipation Abdominal pain		Regurgitation Nausea/vomiting Chronic diarrhea Constipation, Colic Blood in stool Food refusal	Dysphagia Dyspepsia Retrosternal pain Malabsorption Failure to thrive or weight loss
Respiratory	Nasal pruritus Nasal congestion Wheezing Cough Rhino-conjunctivitis	Dyspnea Stridor Chest tightness and nasal discharge	Recurrent pulmonary infiltrates associated with tachypnea and recurrent fevers	
Cutaneous	Urticaria Eczema Angioedema Flushing Pruritus		Atopic dermatitis	
Associated disorders	**IgE-mediated CMPA**		**Non-IgE-mediated/mixed CMPA**	
	I. Systemic IgE-mediated reactions (Anaphylaxis) A. Immediate-onset reactions B. Late-onset reactions II. IgE-mediated gastrointestinal reactions A Oral allergy syndrome B Immediate gastrointestinal allergy III IgE-mediated respiratory reactions A Asthma and rhinitis secondary to ingestion of milk B Asthma and rhinitis secondary to inhalation of milk (e.g., occupational asthma) IV IgE-mediated cutaneous reactions A Immediate-onset reactions 1 Acute urticaria or angioedema 2 Contact urticarial B Late-onset reactions Atopic dermatitis		I Atopic dermatitis A Immediate-onset reactions B Late-onset reactions II Non IgE-mediated gastrointestinal reactions Gastro-esophageal reflux disease (GERD) Crico-pharyngeal spasm Allergic eosinophilic esophagitis (EoE) Severe irritability (colic) Cow's milk-induced iron deficiency anemia Food protein-induced enterocolitis syndrome (FPIES) Food protein-induced allergic proctocolitis (FPIAP) Food protein-induced enteropathy (FPE) III Non-IgE-mediated respiratory reactions Pulmonary hemosiderosis (Heiner syndrome)	

Adapted from Fiocchi et al. (28), Koletzko et al. (14), and Brill (18).

of a food allergy (17). Moreover, in accordance with the dual antigen exposure hypothesis, early consumption of food protein is considered to induce oral tolerance in certain foods (i.e., peanut, egg) (5, 6).

Tertiary prevention in children with established CMPA is based on avoidance of allergenic food and treatments that target tolerance induction. Cow's milk protein exclusion diet (elimination diet) is considered the most effective treatment for CMPA. Maternal breastfeeding is the best strategy with use of extensively hydrolyzed formula (eHF), amino acid-based formula (AAF) or formula that contains soy proteins (after 6 months of age) when breastfeeding is not possible. Recently, implementation of specific oral immunotherapy to achieve an active immune response has been considered in the management of CMPA (**Figure 1**) (17).

Avoidance of Allergenic Food

In exclusively breast-fed infants with CMPA, the continuation of breast-feeding along with maternal elimination diet for CMP containing products may be considered in certain cases with the aid of supplemental calcium and vitamin D (46, 61, 62).

In exclusively breast-fed or formula-fed infants with CMPA, weaning food is recommended to be free of CMP until oral challenge tests, dependent on confirmation of the tolerance (14, 61, 62). The introduction of supplementary foods should not be delayed, although it should occur one food at a time in small amounts and only after the infant is at least 17 weeks of age, preferably while the mother is still breastfeeding (14, 63, 64).

Induction of Tolerance

(a) Hydrolyzed Formulas, Probiotics, and Tolerance Development in CMPA

In a previous study of 260 children with CMPA (aged 1–12 months, IgE-mediated CMA in 42.7%), a food challenge performed after 12 months to assess the acquisition of tolerance indicated that an eHCF supplemented with the probiotic *Lactobacillus rhamnosus* GG (LGG) induced higher tolerance rates (78.9%) than eHCF without LGG (43.6%) and other formulas, including AAF (18.2%), hydrolyzed rice formula (32.6%), and soy formula (23.6%) (65). Binary regression analysis indicated a significant association of the mechanism of CMA and the formula type with tolerance development with a higher

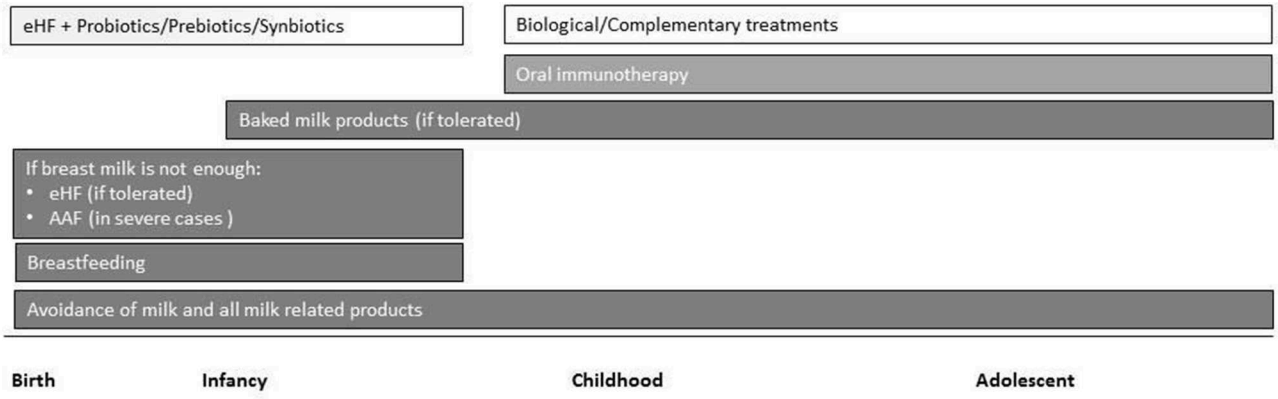

FIGURE 1 | Overview of current and emerging treatment interventions for CMPA in childhood. Intensity of bar color represents the strength of the evidence (eHF, extensively hydrolyzed formula; AAF, amino acid-based formula).

likelihood of acquiring tolerance in subjects with a non-IgE-mediated mechanism and with the use of eHCF and eHCF + LGG. The authors concluded that eHCF accelerates tolerance acquisition in children with CMPA compared with other dietetic choices, and this effect is augmented by LGG (65).

eHCF with LGG was also shown to reduce the incidence of other allergic manifestations and to accelerate the development of oral tolerance in children with IgE-mediated CMPA (66).

Preliminary data suggested immune-regulatory properties of hydrolyzed casein peptides to be associated with a potential long-term effect of dietary intervention with eHCF +LGG on the immune system and tolerance induction via positive effects on gut dysbiosis, short-chain fatty acid production, and epigenetic regulation of Th1 and Th2 cytokine gene expressions (67–69). However, given that the effect of eHF with LGG on tolerance acquisition has been based on a limited number of studies, further RCTs investigating the effect of eHF with pre-probiotics are needed to clarify its role in the development of tolerance in CMA.

(b) Modified Formula

In formula-fed infants, the first elimination diet is an eHF with proven efficacy and cost effectiveness in CMPA (14, 61, 70, 71). An AAF may be considered as the first choice in infants with severe or life-threatening symptoms (**Table 2**) (14, 61).

The risk of reaction or no response to eHF is far below 10% of the infants with uncomplicated CMPA and is more common, reaching up to 40% in patients with a more severe syndrome and multiple food allergies (17, 72–77). AAF provides a safe alternative for individuals who are allergic to eHFs and provides the ability to refine the diagnosis of eHF allergy (72, 73, 78).

Soy protein formula, if tolerated, is an option beyond 6 months of age, whereas it is not indicated in situations of enteropathy or non-IgE-mediated allergies that are sensitive to soy (**Table 2**) (14, 17).

In a systematic review on the efficacy of AAF in relieving the symptoms in CMPA, the analysis has demonstrated that for infants with CMPA who completely tolerate eHF, there was no additional benefit from the use of AAF (79). The authors noted that AAF and eHF were equally effective in resolving gastrointestinal and skin symptoms in uncomplicated CMPA based on evidence from head-to-head randomized controlled trials (RCTs), whereas a clear need for AAF was shown in infants with intolerance to eHF (79). Given that eHF-intolerant patients were also more likely to have severe atopic eczema, anaphylaxis, reflux esophagitis or FPIES and FPIAP with failure to thrive, more severe symptomatology is considered necessary to warrant the use of AAF (4, 6, 73, 75, 79–81).

In patients with CMPA, eHF may be the primary choice when the clinical presentation includes colic, constipation, GERD, acute urticaria, acute angioedema, atopic dermatitis, and gastroenteritis, whereas AAF may be the primary choice in severe cases, such as anaphylaxis, enteropathy, eosinophilic esophagitis and FPIES, as well as cases of multiple system involvement, multiple food allergies, and intolerance to eHF (**Table 2**) (7, 14, 28, 35, 74, 82).

The specific immunologic mechanisms that drive most of the non-IgE-mediated food allergies are largely unknown; therefore, the therapies for these conditions remain non-specific (53). In FPIES, breastfeeding can be continued unless maternal ingestion of a non-identified allergen triggers acute or chronic-FPIES. An eHF is usually well-tolerated, although up to 20% of patients may need an AAF (51). AAF formula was the primary choice in previous guidelines, whereas extensively hydrolyzed casein formula (eHCF) in International FPIES (2017) guidelines is recommended as the first choice. AAF should be considered the first option in FPIES patients with hypoalbuminemia (52). Infants with milk/soy-FPIES may be breastfed, and if the severity of FPIES is mild to moderate, a hypoallergenic formula approved for infants with milk allergy, such as eHCF, may be used (52). A case-based evaluation is necessary until sufficient clinical experience is available.

In FPIAP, elimination of food from the maternal diet is usually sufficient. In rare cases, the identification of the causative factor may be difficult. An eHF or AAF might be necessary if breastfeeding is not an option or if blood in stools becomes severe (**Table 2**) (51).

TABLE 2 | Choice of formula in non–breast-fed infants with confirmed CMPA according to clinical presentation (14, 17, 52, 61).

CMPA Clinical presentation	1st choice	2nd choice	Key points
Anaphylaxis	AAF	eHF	
Immediate gastrointestinal allergy	eHF	AAF	Differential diagnosis of anaphylaxis should be made
Food protein-induced enterocolitis syndrome (FPIES)	AAF/eHF		AAF in previous guidelines, whereas extensively hydrolyzed casein formula (eHCF) in International FPIES (2017) guidelines is recommended as the first choice. A case-based evaluation is necessary until sufficient clinical experience is available. AAF should be considered the first option in FPIES patients with hypoalbuminemia
Atopic dermatitis	eHF	AAF/SF in infants aged > 6 months	AAF should be considered the first choice in breast feeding infants with CMPA or severe atopic dermatitis.
Allergic eosinophilic esophagitis	AAF		
Food protein-induced enteropathy	eHF	AAF	AAF should be considered the first choice in enteropathies accompanied with hypoproteinemia.
Food protein-induced allergic proctocolitis	eHF	AAF	
Milk-induced chronic pulmonary disease (Heiner's syndrome)	AAF	SF in infants aged > 6 months	
Asthma and rhinitis	eHF	AAF/SF in infants aged > 6 months	Anaphylaxis should be ruled out via differential diagnosis May be considered in treatment failure for asthma/rhinitis Solitary presence is rare and often involves other systems
Acute urticaria or angioedema	eHF	AAF/SF in infants aged > 6 months	Anaphylaxis should be ruled out via differential diagnosis
Gastroesophageal reflux disease (GERD)	eHF	AAF	Frequent in infancy. In cases with failure to classical treatment, diagnosis of CMPA should be considered
Constipation	eHF	AAF	Frequent in infancy. In cases with failure to classical treatment, diagnosis of CMPA should be considered
Infantile colic	eHF	AAF	Frequent in infancy. In severe cases, CMPA should be considered in the differential diagnosis.

eHF, extensively hydrolyzed formula; AAF, amino acid-based formula; SF, soya formula.
Adapted from Koletzko et al. (14), Martorell-Aragonés et al. (17), Kansu et al. (61), and Nowak-Wegrzyn et al. (52).

(c) Baked-Milk Products

IgE-antibody production is primarily directed at heat-sensitive conformational epitopes in transient milk allergy, whereas IgE antibodies are also produced against heat-stable sequential epitopes in persistent allergy. Greater IgE-epitope diversity and higher IgE affinity are related to more severe milk allergy (83).

Given the reduction in allergenicity by destruction of conformational epitopes of milk proteins via extensive heating or food processing, children with transient milk allergy are considered likely to tolerate baked-milk products (62, 83). Accordingly, clinical trials indicated that nearly 75% of children with IgE-mediated CMPA tolerate baked milk-containing foods, such as muffins, cakes and bread, and the inclusion of baked milk products in the children's diet is suggested to accelerate the development of unheated-milk tolerance compared to a strict milk avoidance approach, which is currently the standard of care (83–86). Tolerance to baked milk is a marker of transient IgE-mediated cow's milk allergy, whereas reactivity to baked milk portends a more persistent and severe phenotype of CMPA with a higher risk of severe anaphylaxis and a more protracted course (83, 87). The addition of baked-milk to the diet appears to accelerate the development of unheated-milk tolerance compared to strict avoidance, along with a significant increase in casein IgG4 values in the baked-milk-tolerant group, similar to those spontaneously outgrowing milk allergy and treated with milk oral immunotherapy (OIT) (83, 88–92). Recent studies on the regular consumption of baked milk and related immunologic changes reinforce baked milk as a proactive treatment for food allergy (85, 86, 93–95). A randomized controlled trial assessed the effect of baked milk on accelerating tolerance in 84 children with CMPA who tolerated baked milk in oral food challenge (OFC) (96). The tolerance rate of the children in the case group who consumed baked milk products for 1 year was higher than that in the avoidance group (88.1 vs. 66.7%, p:0.018). While the introduction of baked milk into the diet was demonstrated to accelerate tolerance, the initial sIgE levels of milk, casein, and beta-lactoglobulin did not predict the tolerance of unheated cow's milk (96).

Although the ingestion of baked-milk products is considered a form of immunotherapy with more favorable safety, higher convenience, lower cost, and less labor intensity when compared to OIT, a clear need for strict avoidance in a subset of milk-allergic patients is emphasized, since nearly 25% of children were initially baked-milk-reactive. In addition, the likelihood of treatment discontinuation due to reactions to lesser-cooked forms of milk in nearly 10% of children who passed the initial muffin challenge is considered to highlight the challenges of strict adherence to proper food preparation (83).

The consumption of baked milk is suggested to enhance the quality of life by removing unnecessary dietary restrictions and

to change the natural evolution of milk allergy by promoting the development of tolerance to regular cow's milk (83, 85–87, 94).

The "milk ladder" classifies factors associated with the allergic potential of cow's milk food stuffs in terms of volume or quantity, effect of heating and wheat matrix effect from Stage 1 (small quantity, baked and matrix) to Stage 4 (fresh milk products) (**Table 3**). The use of the milk ladder in patients who can tolerate baked milk may facilitate the introduction of less allergic milk products (97, 98).

(d) Oral Immunotherapy

OIT has been applied in treating food allergy for over a century and involves monitored repeated administration of gradually increasing doses of allergen over months to years to enable non-reactivity to foods (desensitization) (22, 83, 91, 92).

The data from meta-analyses indicated a ten times greater probability of tolerance development with OIT compared to a strict elimination diet in patients with IgE-mediated CMPA (99). In addition, a significant reduction in skin test positivity to the food allergen, an increase in the specific IgG4 titers, and a substantially lower risk of reactions to the allergen have been shown among individuals administered OIT (17, 100).

Although OIT may be effective in raising the threshold of reactivity to a range of foods in patients with IgE-mediated food allergy while receiving (desensitization) and post-discontinuation of OIT, it was also associated with an increased risk of local and systemic adverse events (22, 101).

TABLE 3 | Milk ladder: classification of cow's-milk-containing foods* (97, 98).

Stage 1	**Stage 2**	**Stage 3**	**Stage 4**
Baked products (at least 180°C heating and 30-min duration) containing cow's milk protein: - Small crumb of a biscuit containing < 1 g of protein per biscuit - Build up to 1 biscuit over 5 weeks as tolerated.	Other baked products containing cow's milk protein: - Biscuits, - Cakes, - Muffin, - Pie, Butter. Margarine.	Products containing cooked cheese or whole cow's milk as a heated ingredient: - Custard, - Cheese sauce, - Pizza, - Rice pudding, Chocolate. Chocolate-coated items. Fermented desserts, Yogurt, Fromage frais.	Uncooked cheese Uncooked non-yogurt desserts, for example, ice cream or mousse. Cow's milk UHT milk followed by pasteurized milk and then unpasteurized milk (if this form is preferred by the family).

Stage 1: Small quantity, baked, and matrix. Stage 2: Larger quantity, baked and matrix OR traces without matrix, or with minimal heating. Stage 3: Larger quantity, less heating, and less matrix OR all with some degree of protein change with heating or manufacturing. Stage 4: Fresh milk products.

At all stages, start with a small amount, and gradually increase. Each individual product in Stage 3 is to be initially introduced in trace amounts, as they have more milk protein and a lower degree of heat treatment or protein denaturation. There is also variability in milk protein between products. If a reaction occurs, the food that caused the reaction should be stopped, and reintroduction should be continued with food from a lower stage in smaller amounts.

** It is more appropriate to use the milk ladder in non-IgE mediated CMPA; it is not advisable in infants/children with prior anaphylaxis to small amounts of milk, asthma, or very high cow's milk sIgE or large skin prick test wheals.*

The meta-analysis of the Cochrane Collaboration concluded that OIT is effective for inducing desensitization in most patients with IgE-mediated CMPA, whereas albeit mild and self-limiting in most cases, adverse effects are frequently observed, thus limiting its use by selected patients over 3 years of age and in centers with experience in the management of OIT and the capacity to deal with the possible adverse reactions (17, 22, 102).

The most common adverse events related to OIT are observed in the gastrointestinal system, and EoE is of particular concern because of the significant association with OIT. The meta-analysis conducted by Petroni and Spergel determined the incidence of EoE in patients with OIT for milk, egg and peanut (103). The incidence of OIT-related EoE was 5.3% of patients receiving food OIT in the studies that showed the diagnosis of EoE by biopsy findings (104). This observation suggests that food OIT could increase the risk of EoE development, and additional studies investigating the long-term effect of OIT with standard diagnostic procedures for EoE are needed.

CONCLUSION

In conclusion, this review by experts from Turkey aimed to document the current knowledge on tolerance development in infants and children with CMPA to increase the awareness of clinicians concerning the new approaches in CMPA treatment, given the change in the natural history and prevalence of food allergy during the last two decades and the related changes in the guidelines in terms of prevention and tolerance induction strategies in food allergies in recent years. Accordingly, the change in prevention strategies for food allergy has been emphasized in this paper. This change in prevention strategies is based on the lack of evidence to support the efficacy of food allergen avoidance in infancy and the concept of the dual-allergen-exposure hypothesis, which suggests that allergen exposure through the skin leads to sensitization, whereas the early oral consumption of allergenic food protein induces oral tolerance. The early introduction of allergenic foods to the infant's diet in small amounts is considered likely to reduce the development of food allergies through oral tolerance induction.

In exclusively breast-fed infants, continuation of breast-feeding is recommended with elimination of cow's milk and products from the maternal diet when an infant reacts to the amount of milk protein passed on from maternal consumption during breastfeeding. If breastmilk is not sufficient, the use of supplemental formula should be considered in CMPA. No delay in the introduction of complementary feeding in infants with CMPA is recommended. Nearly 75% of children with IgE-mediated CMPA tolerate baked-milk-containing foods; thus, the consumption of baked milk could change the natural evolution of milk allergy by promoting the development of tolerance to regular cow's milk and enhancing the quality of life by removing unnecessary dietary restrictions.

The risk of reaction or no response to eHF is far below 10% of infants with uncomplicated CMPA and is as common as 40% in those with a more severe syndrome and multiple food allergies. Accordingly, if a supplemental formula is required,

an eHF or AAF may be utilized. This decision must be made on an individualized basis, with use of an eHF with proven efficacy in CMPA as the first elimination diet in formula-fed infants with CMPA. However, AAF should be used in severe cases, such as anaphylaxis, enteropathy, EoE and FPIES, as well as cases of multiple system involvement, multiple food allergies, severe atopic dermatitis, and intolerance to eHF. The use of eHF with the supplementation of the probiotic LGG shows promising results on the early acquisition of tolerance in mild cases of cow's milk allergy. Additional studies investigating the effect of eHF with pre-probiotics will clarify its role in the development of tolerance in CMA. Although OIT is effective in raising the threshold of reactivity to a range of foods in patients with IgE-mediated food allergy, the use of OIT should be restricted to selected patients over 3 years of age and in a center with experience in the management of OIT, given the increased risk of local and systemic adverse events.

Finally, the tolerance development seems to be a relatively new concept in CMPA, inducing a shift in the treatment of CMPA from a passive (avoidance of responsible allergen) toward a proactive (tolerance induction) strategy. However, it should also be kept in mind that currently there is no evidence-based protocol for the strategy of tolerance in most children with CMPA, and further studies are needed. Given the recently described different clinical phenotypes of food allergy, it seems necessary to adopt an individualized nutrition and treatment algorithm that is tailored to each individual's needs and medical conditions in the management of CMPA.

AUTHOR CONTRIBUTIONS

CS had primary responsibility for the manuscript preparation. All authors have read and approved the final manuscript.

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Metagenome of Gut Microbiota of Children With Nonalcoholic Fatty Liver Disease

Yuzhen Zhao[1,2†], Jianli Zhou[1†], Jiaqi Liu[1], Zhaoxia Wang[1], Moxian Chen[1] and Shaoming Zhou[1*]*

[1] Division of Gastroenterology, Shenzhen Children's Hospital, Shenzhen, China, [2] Shantou University Medical College, Shantou, China

Aim: To investigate the intestinal flora of nonalcoholic fatty liver disease (NAFLD) in Chinese children and adolescents using metagenomic approach.

Methods: All participants underwent magnetic resonance spectroscopy (MRS) to quantify liver fat content. Hepatic steatosis was defined as MRS proton density fat fraction (MRS-PDFF) >5%. A total of 58 children and adolescents were enrolled in this study, including 25 obese NAFLD patients, 18 obese non-NAFLD children, and 15 healthy children. Stool samples were collected and analyzed with metagenomics. We used Shannon index to reflect the alpha diversities of gut microbiota. Wilcoxon rank sum test and Kruskal-Wallis test were performed to evaluate alpha diversities between groups. At last, the differences of gut microbiota composition and functional annotations between obese with and without NAFLD and healthy children were assessed by Kruskal-Wallis test.

Results: Significant differences in gut microbiota composition and functional annotations among three groups of children and adolescents have been observed. Deep sequencing of gut microbiota revealed high abundance of phylum *Proteobacteria* (*Gammaproteobacteria*) in obese NAFLD patients, comparing with the control group. Overall, obese children without NAFLD had less abundant *Helicobacter* and *Helicobacter pylori*. Compared to the control group, in obese children with NAFLD, the abundance of *Bacteroidetes* (*Alistipes*) were significantly reduced. *Faecalibacterium prausnitzii* was the only species representing a difference between obese children with and without NAFLD. There were not significant differences in terms of alpha diversity among three groups. Functional annotations demonstrated that several pathways were differentially enriched between groups, including metabolism of other amino acids, replication and repair, folding, sorting, degradation, and glycan biosynthesis and metabolism.

Conclusion: Significantly differences are observed in gut microbiota composition and functional annotations between obese children with and without NAFLD in comparison to the healthy children group. The characteristic of gut microbiota in this study may contribute to a further understanding the gut-liver axis of pediatric NAFLD in China.

Keywords: nonalcoholic fatty liver disease, gut microbiota, metagenome, children and adolescents, obese

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***Correspondence:**
Moxian Chen
cmx2009920734@gmail.com
Shaoming Zhou
zhousm15d@aliyun.com

† These authors have contributed equally to this work

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) has emerged as one of the most common chronic liver disorders in children and adolescents, affecting 5–10% of the general child population, and 28–41% of obese children (1). The term NAFLD is also known as a broad spectrum ranging from simple steatosis, nonalcoholic steatohepatitis (NASH), to hepatic cirrhosis. In addition to liver-related disorders, NAFLD is also revealed as a risk factor of chronic kidney disease, cardiovascular diseases, obesity, and diabetes (2–4). What is more, 20% of NASH cases may progress to hepatic cirrhosis, and eventually liver failure (5). Therefore, NAFLD is of great importance on public health, especially in children and adolescents.

The human intestinal tract is the main habitat of microbiota, with $\sim 10^{14}$ bacteria existing, which comprises of 100 times more genes than humans. As a group of symbiotes, gut microbiota play an important role in metabolic and immune balance of human beings. First, gut microbiota participate in digestion and absorption of nutrients and production of vitamins and minerals. Second, gut microbiota can affect the production of intestinal hormones such as glucagon-like peptide-1, thereby affecting the metabolism of the host (6). In recent research, we learn that gut microbiota is closely related to the occurrence and development of NAFLD through Gut-liver axis (7, 8). Increasing evidence suggests that gut microbiota connect with hepatic steatosis in several ways (9–11): (1) it affects the appetite signal of the host; (2) it can also increase energy extraction from the intestine; (3) the metabolism of bile acids change, therefore affecting fats and lipid-vitamins obtained in the intestine; (4) the metabolism of choline is affected; (5) it also contributes to increased inflammation in host organisms; (6) intestinal bacterial overgrowth and intestinal permeability increase will lead to bacteria translate into the systemic circulation and endotoxemia.

As early as 2004, Backhed et al. first connected gut microbiota with obesity and NAFLD (12). They found that gut microbiota could not only affect the absorption and storage of energy, but also stimulate the production and infiltration of triglycerides in hepatocytes. Mouzaki et al. (11) diagnosed NAFLD with liver biopsy and further detected the composition of gut microbiota. They demonstrated that gut microbiota in healthy controls were varied from patients with simple fatty liver disease and NASH. The *Bacteroides* in NASH patients were significantly lower compared to that in simple fatty liver disease patients and healthy controls, and were independent from diet and BMI. While Raman et al. (13) and Li Fan et al. showed that compared with healthy controls, the differences in intestinal flora of NAFLD mainly occurred at family and genus levels, of *Firmicutes* (14).

In recent years, with increasing prevalence of obesity and NAFLD in children and adolescents, drawing more attention of researchers to these populations. Zhu et al. performed 16S RNA sequencings to analyze the composition of gut microbiota in Caucasian children in a controlled diet with NASH, obesity, and healthy controls in the United States (15). They suggested that alcohol-producing bacteria *Escherichia* was significantly increased in NASH children. Comparing with healthy controls, children with NASH and obesity had increased *Bacteroidetes* and decreased *Firmicutes*. The differences of gut microbiota between NASH patients and obese children were exhibited in *Proteobacteria*, *Enterobacteriaceae,* and *Escherichia*. Michail et al. (16) conducted metagenomics and proteomics to examine the composition, function, and metabolism of intestinal flora in obese children with and without NAFLD against the healthy children controls. *Gammaproteobacteria*, *Prevotella* and ethanol were observed to have a significant increase in NAFLD patients. In addition, there was an increase in the pathways involved in energy metabolism and lipid metabolism in children with NAFLD, compared with the healthy controls. Researchers in Italy (5) analyzed the gut microbiota in children with simple fatty liver disease, NASH, obesity, and healthy children but got a different result. Compared with healthy groups, *Actinobacteria* were increased in NAFLD children, while *Bacteroidetes* were decreased, which was contrary to the research by Zhu et al. (15). There was no significant difference in gut microbiota composition among the groups of children with simple fatty liver disease, NASH, and obesity, which was consistent with the previous study by Zhu et al.

Researchers have demonstrated that gut microbiota of NAFLD patients could undergo a compositional and functional change, but the differences of gut microbiota in obesity, NAFLD, and healthy children have no consistency. Furthermore, studies are rarely reported in the case of Chinese pediatric NAFLD. Therefore, the present study used metagenome to clarify the composition and function of gut microbiota in obese children with and without NAFLD against healthy children in China in order to unravel the relationship of liver-gut axis and its function in children NAFLD.

MATERIALS AND METHODS

Subjects

The research was approved by the Human Ethics Committee of Shenzhen Children's Hospital (Supplemental Document Ethic Committee Approval). A total of 58 participants were recruited in the study during May 2017 to July 2018, including 43 obese [body mass index (BMI) above age- and gender-specific 95th percentile] and 15 healthy controls. The average age of participants is 13.7 years, ranging from 9 to 17 years. In view of their diagnosis in our previous study (17), children and adolescents were stratified into obesity with ($n = 25$) or without ($n = 18$) NAFLD, and matched healthy controls ($n = 15$). All subjects with antibiotics or probiotics history in the past 3 months were exclude. All authors of this paper had access to the study data and reviewed and approved the final manuscript.

Abbreviations: BMI, body mass index; KEGG, kyoto encyclopedia of genes and genomes; KOs, KEGG orthologies; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; MRS, magnetic resonance spectroscopy; PDFF, proton density fat fraction.

Magnetic Resonance Spectroscopy (MRS)

Children and adolescents underwent single-voxel MRS scanning using 3.0 T MR unit (MAGNETOM Skyra, Siemens Healthcare, Erlangen, Germany) in accordance with previous research (17). Hepatic steatosis is defined as MRS proton density fat fraction (MRS-PDFF) > 5%.

Fecal Sample Collection, DNA Extraction and Metagenomic Sequencing

Before collecting, methods and notes were explained by researchers. Samples were collected using a sterile kit and frozen at −80°C immediately until detection.

All stool samples were analyzed in Beijing Genomics Institute (BGI, Shenzhen, China). Briefly, DNA was isolated from 58 fecal samples and an average of 5.92 Gb of sequence was obtained from each subject. All DNA fragments were purified by QIA quick PCR Purification Kit (Qiagen) during library construction. Agilent 2100 Bioanaylzer and ABI StepOnePlus Real-Time PCR System were used to qualify and quantify the sample libraries. The qualified libraries were then sequenced using Illumina HiSeq System.

TABLE 1 | Characteristics of all subjects.

	Obese NAFLD (n = 25)	Obesity (n = 18)	Health controls (n = 15)
Male	19 (76.0%)	12 (66.7%)	10 (66.7%)
Female	6 (24%)	6 (33.3%)	5 (33.3%)
Age (years)	14.1 ± 2.1	13.9 ± 1.3	13.7 ± 2.0
BMI (kg/m^2)	30.6 ± 3.4*	28.6 ± 1.8*	20.2 ± 1.9
MRS-PDFF	15.5% ± 8.8%	2.8% ± 1.2%	1.3% ± 0.26%

*$P < 0.05$ versus healthy controls.

Metagenomic Analysis

In order to acquire accurate sequences, we removed sequences with a 90% similarity to the human genome. Then we assembled all samples to obtain reads which were more than 300 bp for further analysis. With CD-Hit software (18), genes were combined and clustered. Finally, we got 2,053,172 non-redundant genes. All these genes were blasted against with Kyoto Encyclopedia of Genes and Genomes (KEGG) databases using the Diamond software (19) to obtain function annotations. The taxonomic composition was performed using MEGAN software (20) based on NR databases. Alpha diversity was calculated using Shannon index based on species profile.

Statistical Analysis

All data were statistically analyzed using SPSS 22.0 software and R environment. Numerical variables were firstly tested for normality. Age and BMI between three groups were compared by independent-samples T-test. The Chi-square test was performed for gender comparison between groups. The differences between groups in taxonomic composition and function annotations were determined using Kruskal-Wallis test. Alpha diversity was calculated based on Shannon index. Wilcoxon Rank-Sum test or Kruskal-Wallis test were used for diversity differential analysis, depending on group numbers.

RESULTS

Clinical Characteristics

A total of 58 participants were enrolled in the present study with an average age of 13.7 ± 1.8 years (range 9–17 years). According to MRS examination, hepatic steatosis was diagnosed with MRS-PDFF > 5%. We divided all subjects into three groups based on liver fat content and BMI. Among them were obese NAFLD patients (n = 25), obese patients (n = 18), and healthy controls (n = 15). There was no difference in gender and age among

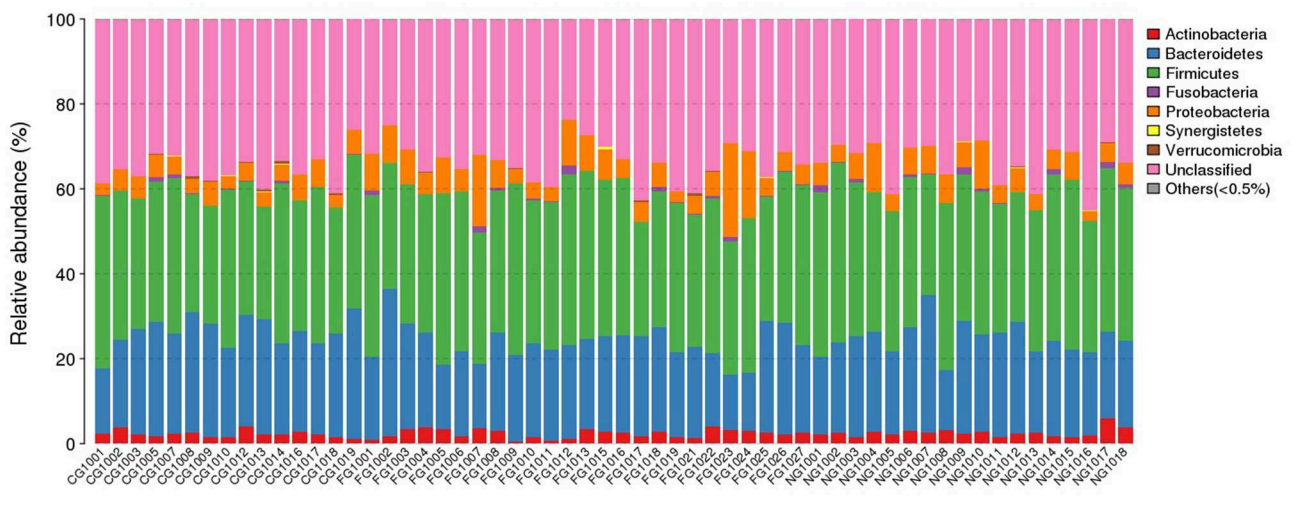

FIGURE 1 | Phylum distribution of gut microbiota of all subjects. The distribution of bacterial phyla (abundance >0.5%) of each individual is presented as bar chart in terms of percentage weight.

these three groups. The basic information of these three groups is summarized in **Table 1**.

Taxonomy Comparison of Gut Microbiota at Phylum Level

Taxonomic composition of all subjects was analyzed at phylum, class, order, family, genus, and species level. To obtain an accurate taxonomic change in all samples, abundant levels with <0.5% average abundance were classified into others. We observed significant differences between obese patients with and without NAFLD and healthy controls. At phylum level, we found that *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* were the dominant phyla in all samples (**Figure 1**). Comparing with the healthy controls, the abundance of *Proteobacteria* was statistically increased and *Bacteroidetes* was decreased in obese NAFLD children. In contract, *Firmicutes* and *Actinobacteria* did not show large variations in three groups of children (**Figure 2**).

Taxonomy Comparison of Gut Microbiota at Class, Genus, and Species Level

Within phylum *Firmicutes*, class *Negativicutes*, genus *Phascolarctobacterium,* and species *Phascolarctobacterium succinatutens* exhibited a significant increase in obese NAFLD children, compared with the healthy controls (**Supplemental Table 1**). In contrast, genus *Lactobacillus*, *Oscillibacter,* and *Ruminiclostridium* showed a strong decrease in obese NAFLD patients. Additionally, we observed that *Faecalibacterium prausnitzii* was the only species representing differences between obese children with and without NAFLD. The Pathogenic genus *Clostridium* showed no statistical difference among these groups.

Decreased abundance of *Bacteroidetes* was mainly due to the decreased abundance in class *Bacteroidia*, genus *Alistipes,* and *Paraprevotella* in the obese children with or without NAFLD (**Supplemental Table 1**). Besides, at species level, we found that the abundance of *Bacteroides clarus* and *Odoribacter splanchnicus* were reduced in obese children groups with and without NAFLD. Interestingly, *Parabacteroides johnsonii* only exhibited a decrease in the obese NAFLD group, compared with the healthy group.

The increased representation of *Proteobacteria* in obese NAFLD group was mostly explained by the increased class *Gammaproteobacteria*, genus *Klebsiella*, *Kluyvera,* and species *Klebsiella pneumoniae* and *Kluyvera ascorbata* (**Supplemental Table 1**). It is worth noting that genus *Helicobacter* and species *Helicobacter pylori* were present and decreased in obese non-NAFLD children, compared with the healthy control group.

Furthermore, it is noteworthy that there were not any differences in phylum *Actinobacteria* in the three groups of children, which was in contrast to previous studies (15).

Ecological Diversities of Gut Microbiota

In the present study, we used Shannon index to assess the Alpha diversity of the community. Three groups of subjects did not demonstrate statistical difference in diversity (**Table 2**).

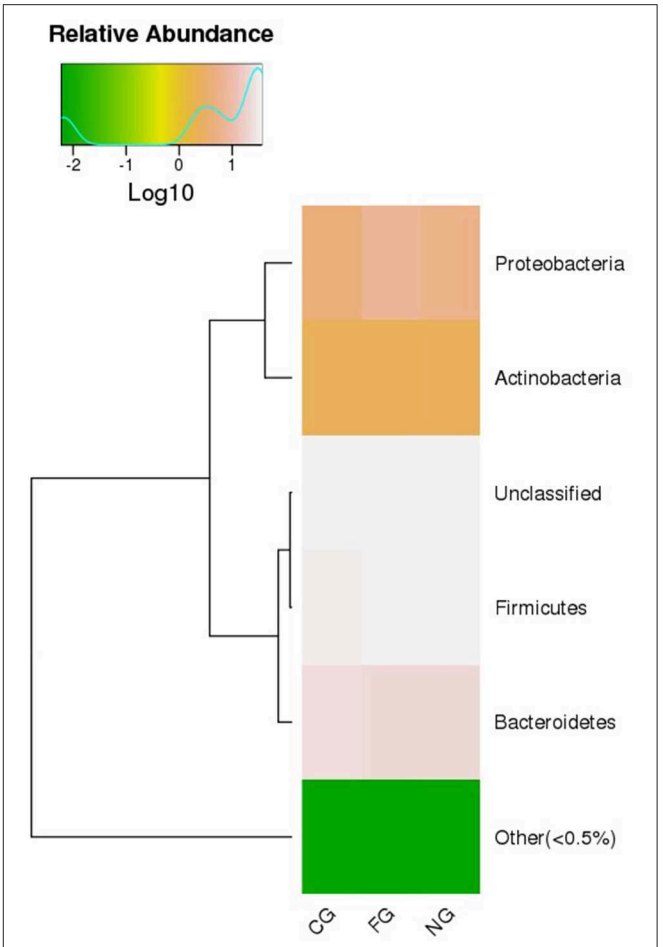

FIGURE 2 | Heat map of gut microbiota between groups at phylum level. Group comparison and clustering of major bacteria phyla and their relative abundance. CG, healthy controls; FG, obese NAFLD group; NG, obese non-NAFLD group.

TABLE 2 | Comparison of Shannon-index between groups.

Group	Statistical method	*P*-value
FG vs. CG vs. NG	Kruskal-Wallis	0.3
FG vs. NG	Wilcoxon Rank-Sum	0.6
FG vs. CG	Wilcoxon Rank-Sum	0.1
CG vs. NG	Wilcoxon Rank-Sum	0.3

CG, healthy controls; FG, obese NAFLD group; NG, obese non-NAFLD group.

Functional Annotations of Gut Microbiota

Subsequently, we further assessed functional annotations of gut microbiota via Diamond software against pathway information from the KEGG database. In total, by using Wilcoxon rank-sum test, we observed 923 KEGG orthologies (KOs) in three groups (**Figure 3**). Compared to healthy controls, pathways such as replication and repair and metabolism of other amino acids in KEGG levels 2 were significantly decreased in obese children with and without NAFLD (**Figures 4A,C**). The folding, sorting,

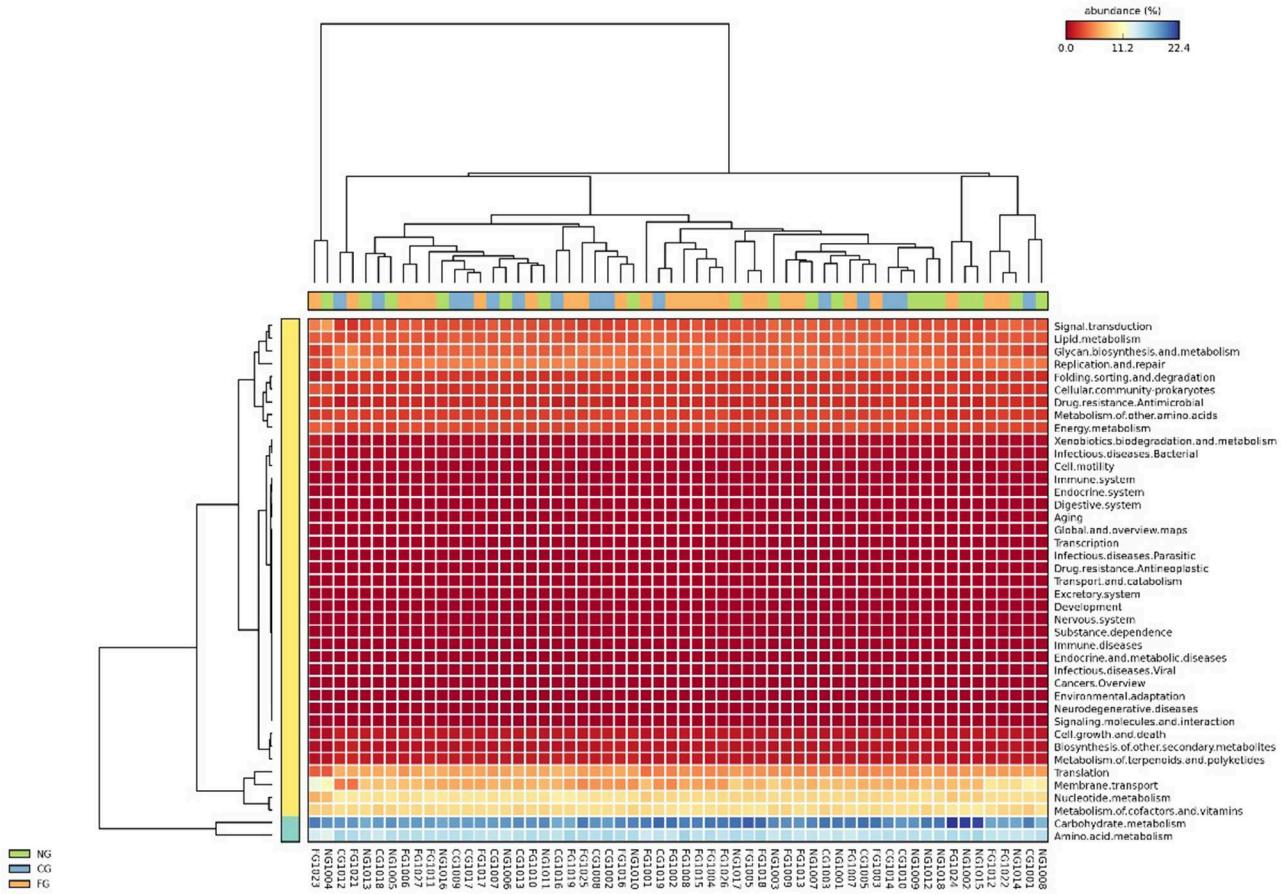

FIGURE 3 | Heat map of gut microbiota KEGG functional annotations. The KEGG pathway annotation was carried out for each individual sample. The abundance of each KEGG term was then clustered to represent as a heatmap.

and degradation pathway decreased in obese patients as well, in comparison to the healthy group. Furthermore, pathways like the digestive system, immune system, glycan biosynthesis, and metabolism showed considerable differences between the two obese groups, with higher abundance in the group of obese NAFLD patients (**Figure 4B**).

DISCUSSION

In the present study, we have assessed gut microbiota of obese children with and without NAFLD in comparison to healthy controls. With metagenomic analysis, we learnt that microbial composition of three groups was significantly different at levels of phylum, class, genus, and species. At phylum level, obese NAFLD children exhibited increased *Proteobacteria* and decreased *Bacteroidetes*, compared to the healthy group, while *Firmicutes* showed no difference between groups. Dating back to 2006, Turnbaugh et al. (21) found that the ratio of *Firmicutes* to *Bacteroidetes* increased in obese mice. In their research, they came to the hypothesis that *Firmicutes* were a group of microbiomes relating to obesity. Genes related to the metabolism of fat and indigestible polysaccharides were detected in *Firmicutes*. In contrast, the population of *Bacteroidetes* is strongly increased in lean mice and humans, suggesting its representative role of body composition (22). The ratio of *Firmicutes* to *Bacteroidetes* also increased in our study. Thus, we postulated that the ratio of *Firmicutes* to *Bacteroidetes* could be a potential biomarker of NAFLD.

In *Bacteroidetes*, compared to the healthy group, genus *Alistipes* (associated with metabolism of plant cell wall polysaccharides and resistant starch) were decreased in obese NAFLD patients. It was in line with research by Zhu et al. (15) and Jiang et al. (23). In compensated and decompensated cirrhosis patients, the abundance of *Alistipes* decreased as well (24). Thus, *Alistipes* may be a group of beneficial microbiomes.

Within *Proteobacteria*, *Gammaproteobacteria* were observed to be increased in obese NAFLD patients in this study. *Gammaproteobacteria* are thought to be involved in choline metabolism and might be inhibited in high levels of choline (25). Besides, the relative abundance of *Gammaproteobacteria* is negatively correlated with liver fat content. However, a contrasting result was present in our study, which may due to

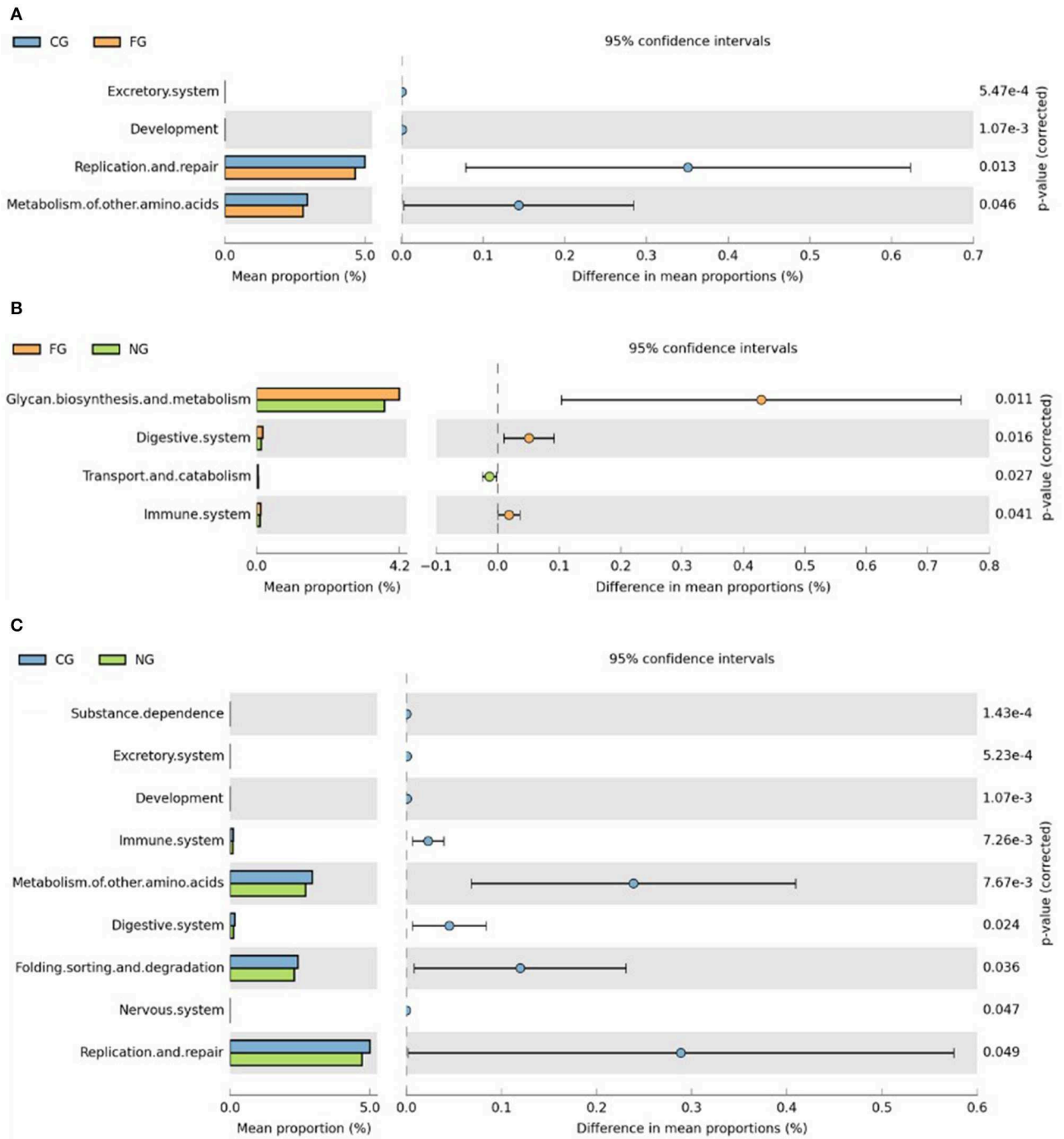

FIGURE 4 | KEGG category comparisons among three groups of children. **(A)** Differences between obese NAFLD and healthy controls in KEGG functional annotations. **(B)** Differences between obese patients with and without NAFLD in KEGG functional annotations; **(C)** Differences between obese non-NAFLD and healthy controls in KEGG functional annotations. CG, healthy controls; FG, obese NAFLD group; NG, obese non-NAFLD group.

the differences in age, religion, and diet of the test subjects. We hypothesized that dietary habits are related to the composition of gut microbiota. Therefore, we can restrict the diet in all subjects for further analysis. Furthermore, genus *Helicobacter* and species *Helicobacter pylori* of *Proteobacteria* were found to be differentially accumulated between obese and healthy children. Researchers in Korea (26) and China (27) found that *Helicobacter pylori* infection was closely related to the development of NAFLD based on large population research. Eradication of *Helicobacter pylori* could be a new method to deal with NAFLD. However,

many studies do not find any connection between *Helicobacter pylori* infection and NAFLD (28, 29). Similarly, we did not observe significant changes between NAFLD patients and healthy children, either. Differently, in this study, we directly detected the *Helicobacter pylori* from stool samples, while most of the above studies detected *Helicobacter pylori* via serum antibody or urea test. Therefore, further study is needed to assess the relationship of NAFLD and intestinal *Helicobacter pylori*.

Although there was no significant difference in *Firmicutes* between three groups in this study. The abundance of probiotic *Lactobacillus* and anti-inflammatory species *Faecalibacterium prausnitzii* have been reported to be decreased in obese NAFLD children. Besides, the abundance of *Faecalibacterium prausnitzii* has been observed to be decreased in NASH patients (15, 30). To our knowledge, *Faecalibacterium prausnitzii* belong to beneficial microbiomes *Clostridium*, which can be developed into a potential kind of probiotic. The ecological imbalance of intestinal *Faecalibacterium prausnitzii* is closely related to inflammatory bowel disease, irritable bowel syndrome and type 2 diabetes (31, 32).

In the current study, ecological diversities of gut microbiota in three groups of subjects did not indicate differences. The same consequence was observed in study by Zhu et al. (15). Gut microbial alpha diversity between NASH patients, obese children and healthy controls were not statistically different. While Del Chierico et al. (5) conducted metagenomic analysis to assess gut microbiota of patients with simple fatty liver disease and NASH, obese, and healthy children. They found that the degree of alpha diversity was highest in healthy children, followed by obese children, NASH children, and simple fatty liver disease patients. Therefore, further study is necessary to analyze the ecological diversities in NAFLD.

Through functional annotations, we observed that several pathways were differentially enriched among these groups, including metabolism of other amino acids, replication and repair, folding, sorting, degradation, and glycan biosynthesis, metabolism and so on, whereas pathways of carbohydrate, lipid, and amino acid metabolism showed no differences. Further investigation of enriched gene clusters may be required to unravel the potential functional module related to obesity or NAFLD. In addition, researchers in Italy have found that urinary metabolomics of obese and NAFLD children are significantly different (33). Therefore, we can also analyze the urinary metabolomics of these children to further explore the metabolism of Chinese children with NAFLD.

The current study has some limitations in experimental design and data analysis. First, the number of children and adolescents studied was relatively small. It was difficult to restrict the same diet in all subjects, which may affect the composition and metabolism of gut microbiota. Second, we assessed gut microbiota via fecal samples instead of intestinal samples. It could not reflect all gut microbiota in the intestine exactly. However, lack of any non-invasive methods to directly obtain samples in the intestine could be the main cause. Therefore, we chose fecal samples to represent gut microbiota. Third, we did not evaluate serum metabolic markers as we described in a previous study (17). Further investigation is needed to assess the connection between gut microbiota and these serum factors.

CONCLUSION

In conclusion, large variations of the composition and functional annotations of gut microbiota of NAFLD patients, obese, and healthy children exist in Chinese children and adolescents. The findings in our study and other studies are noteworthy in the understanding of gut microbiota in pediatric NAFLD. Further analysis is necessary to reveal the consistent relationship and molecular mechanism of gut microbiota in NAFLD pathogenesis.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) under Bioproject PRJNA578215 and will be released upon the publication of this article.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Human Ethics Committee of Shenzhen Children's Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

SZ designed experiments. YZ, JZ, JL, MC, and SZ collected the data and performed the research. YZ, JZ, ZW, and SZ analyzed the data. YZ and MC wrote the manuscript. SZ critically reviewed and revised the manuscript for final submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2019.00518/full#supplementary-material

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The Role of Protein and Free Amino Acids on Intake, Metabolism, and Gut Microbiome: A Comparison Between Breast-Fed and Formula-Fed Rhesus Monkey Infants

Xuan He[1], *Jennie Sotelo-Orozco*[1], *Colin Rudolph*[2], *Bo Lönnerdal*[1] *and Carolyn M. Slupsky*[1,3]*

[1] Department of Nutrition, University of California, Davis, Davis, CA, United States, [2] Mead Johnson Nutrition, Evansville, IN, United States, [3] Department of Food Science and Technology, University of California, Davis, Davis, CA, United States

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Alexandra Papadopoulou, Children's Hospital Hagia Sophia, Greece

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***Correspondence:**
Carolyn M. Slupsky
cslupsky@ucdavis.edu

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Background: Compared to breast-fed (BF), formula-fed (FF) infants exhibit more rapid weight gain, a different fecal microbial profile, as well as elevated serum insulin, insulin growth factor 1 (IGF-1), and branched chain amino acids (BCAAs). Since infant formula contains more protein and lower free amino acids than breast milk, it is thought that protein and/or free amino acids may be key factors that explain phenotypic differences between BF and FF infants.

Methods: Newborn rhesus monkeys (*Macaca mulatta*) were either exclusively BF or fed regular formula or reduced protein formula either supplemented or not with a mixture of amino acids. Longitudinal sampling and clinical evaluation were performed from birth to 16 weeks including anthropometric measurements, intake records, collection of blood for hematology, serum biochemistry, hormones, and metabolic profiling, collection of urine for metabolic profiling, and collection of feces for 16s rRNA fecal microbial community profiling.

Results: Reducing protein in infant formula profoundly suppressed intake, lowered weight gain and improved the FF-specific metabolic phenotype in the first month of age. This time-dependent change paralleled an improvement in serum insulin. All lower protein FF groups showed reduced protein catabolism with lower levels of blood urea nitrogen (BUN), urea, ammonia, albumin, creatinine, as well as lower excretion of creatinine in urine compared to infants fed regular formula. Levels of fecal microbes (*Bifidobacterium* and *Ruminococcus* from the Ruminococcaceae family), that are known to have varying ability to utilize complex carbohydrates, also increased with protein reduction. Adding free amino acids to infant formula did not alter milk intake or fecal microbial composition, but did significantly increase urinary excretion of amino acids and nitrogen-containing metabolites. However, despite the lower protein intake, these infants still exhibited a distinct FF-specific metabolic phenotype characterized by accelerated weight gain, higher levels of insulin and C-peptide as well as elevated amino acids including BCAA, lysine, methionine, threonine and asparagine.

Conclusions: Reducing protein and adding free amino acids to infant formula resulted in growth and metabolic performance of infants that were more similar to BF infants, but was insufficient to reverse the FF-specific accelerated growth and insulin-inducing high BCAA phenotype.

Keywords: infant, formula-feeding, breastfeeding, low protein formula, metabolomics, microbiome

INTRODUCTION

It is well-established that breast-fed (BF) infants exhibit different metabolic outcomes compared to formula-fed (FF) infants. This difference during early development is believed to influence the likelihood of developing health problems later in life such as overweight/obesity and diabetes (1–3). Although the detailed mechanism has not been fully elucidated, disruption of early-life gut microbiota may precede the development of obesity during childhood (4–6). In addition, the high protein content in infant formula has been shown to be a factor that is responsible for stimulation of higher insulin and insulin growth factor 1 (IGF-1) leading to rapid weight gain, while disfavoring the use of fat via lipolysis [the "early protein hypothesis" (7)].

Despite the differences in nutrient composition between breast milk and infant formula, bottle-feeding is recognized as a parent-led process that may lead to habitual overfeeding. FF infants tend to consume significantly greater volumes than BF infants (8, 9). However, it is not completely understood if this is due to feeding mode alone, the different nutrient composition between infant formula and human milk, or if human milk plays a role in how infants regulate intake. Non-human primates have been recognized as a valuable model for controlled dietary studies due to their similarity to humans with regard to neurobehavioral and metabolic development. Rhesus infants can be fed directly from birth *ad libitum* from a bottle resulting in demand-driven, self-regulating feeding. An advantage of this is that it allows for control of external feeding cues.

While free amino acids comprise approximately 5% of the total amino acid content in human milk (10), they are significantly lower in infant formula. Free amino acids in human milk provide for rapid absorption and utilization compared to protein-derived amino acids, and may lead to a different response of BF infants compared to FF infants. For example, glutamate, which is the free amino acid with highest concentration in breast milk (11), was shown to promote satiation and satiety as well as reduce total intake volume and energy consumed when added to infant formula (12). Thus, the presence of amino acids, in addition to the amount of protein in infant formulas may impact metabolic response. However, whether there is an interplay between the addition of free amino acids, intake and overall metabolism still remains to be investigated.

In this study, we used the rhesus monkey as a model to evaluate the physiological response and metabolic consequences of providing a formula with protein content that is slightly lower than the level present in rhesus monkey milk. We further tested whether reducing the protein level and incorporating free amino acids in the formula would decelerate weight gain, provide better support for self-regulation of energy intake, and result in a more similar physiological and metabolic response when compared to a BF reference group. We hypothesized that as the amount of protein and free amino acids in infant formula were modified to become more similar to the mother's milk, phenotypic improvement and a difference in fecal microbial profile would be observed. To investigate the time-dependent response after modulating protein and free amino acids composition in a cow milk-based infant formula on infant metabolism and gut microbiota, longitudinal sampling and clinical evaluation of 30 infant rhesus monkeys were performed from birth to 16 weeks of age. The current study compared the comprehensive metabolic implications of formula- and breast-feeding using an amino acid analyzer (AAA) and nuclear magnetic resonance (NMR) spectroscopy to characterize metabolic fingerprints from serum and urine, in combination with anthropometric measurements, intake records, serum hematology and biochemistry, metabolic hormones, and 16s rRNA fecal microbial community profiling.

MATERIALS AND METHODS

Animals and Diets

Newborn rhesus monkeys (*Macaca mulatta*) in this study were randomly assigned to consume either mother's milk or one of four types of infant formula (regular formula, reduced protein formula, with or without addition of free amino acids that included alanine, glutamate, glutamine, taurine) from birth until 4 months of age ($n = 6$ female monkeys per dietary group). All monkeys were under constant care of nursery and vivarium staff. BF rhesus infants were exclusively breast-fed by their mothers and maintained outdoors. FF rhesus infants were housed individually in polycarbonate isolates with a surrogate mother (a terrycloth dummy) for the first month of life, and then matched with another monkey from the same group and housed in pairs for the reminder of the study. Throughout the study, infant monkeys were only separated briefly from their mother or their pair for sample collection.

After birth, the FF rhesus infants were hand-fed using a nursing bottle every 2 h until 5 days of age, where they progressed to use self-feeder for *ad libitum* access to the study formula. Animal care staff were blinded to the formula. Self-feeding training started the day after birth by placing the rhesus infants next to the feeder nipple with their surrogate mother and gently held in place while they self-fed. FF infants were not offered any monkey chow during the study. Fruits (banana and apple) were given in limited amounts after 3 months to allow them to explore and develop interest in novel foods. After 4 months of this study, all monkeys were returned to the colony. The study

was conducted at California National Primate Research Center (CNPRC) in accordance with Department of Agriculture Animal Welfare Act. The study protocol was approved by University of California, Davis, Institutional Animal Care and Use Committee.

Experimental cow-milk infant formula was produced by Mead Johnson Nutrition (Evansville, IN, USA). Both regular and reduced protein formula contained 80% whey, 20% casein, 5.4 g of fat per 100 kcal. Regular formula contained 2.1 g protein and 11.1 g carbohydrate per 100 kcal. Reduced protein formula contained 1.8 g protein and 11.4 g carbohydrate per 100 kcal. Four amino acids were added to either the regular formula or reduced protein formula, to reach a target of 23 mg glutamate, 8.6 mg glutamine, 2.6 mg alanine, and 7.3 mg taurine per 100 kcal. Infant formula was prepared fresh by mixing 134 g of dry formula with 897 ml of water to make 1 L of formula to achieve final concentrations as shown in **SI Table 1**. Since the amount of added free amino acids was relatively low, the difference in the overall protein content (as evaluated by nitrogen content) was <2%.

Sample Collection

Weight (g), crown-rump length (cm) and biparietal diameter (mm) were recorded at birth and every 2 weeks thereafter. Intake (mL/day) was recorded daily for the FF groups from birth to the end of the study. According to clinical practice and infant care standards, all rhesus infants were fed frequently and on demand, therefore, they were not specifically fasted prior to blood collection. Blood samples (1–3 mL) were drawn monthly via femoral venipuncture to a serum separator tube while hand-restrained. Samples were allowed to clot at room temperature for 30 min followed by centrifugation. Urine and fecal samples were collected biweekly (typically for <20 min but up to 4 h) using a specially designed metabolic unit as previously described (13). A summary of sample collection times is provided in **Figure 1A**.

^{1}H NMR Metabolomics Analysis of Serum and Urine

Sample preparation and data acquisition were virtually identical to our previous monkey work (14). In brief, serum samples were filtered through a 4 kDa (3,000 MW) cut-off centrifugal filter (Amicon, Millipore, Billerica, MA) to remove macromolecules. The filtrate and urine were then prepared for analysis by addition of an internal standard containing 3-(trimethylsilyl)-1-propanesulfonic acid-d6 (DSS-d6) and 0.2% NaN3 in 99.8% D_2O. The pH of each sample was adjusted to 6.8 ± 0.1 by adding small amounts of NaOH or HCl. After loading samples into NMR tubes, samples were run on a Bruker 600 MHz NMR spectrometer and acquired using the NOESY-presaturation pulse sequence (noespyr) at 25°C with water saturation during the 2.5 s prescan delay, a mixing time of 100 ms, 12 ppm sweepwidth, 2.5 s acquisition time, 8 dummy scans and 32 transients.

Quantified metabolites were derived from targeted profiling analysis using Chenomx NMRSuite (Chenomx Inc., Edmonton, Canada). All FIDs were multiplied by an exponential weighting function corresponding to a line broadening of 0.5 Hz. Spectra were manually phased, baseline-corrected and referenced to the DSS-d6 singlet at δ 0 ppm. All compounds in the database have been verified against known concentrations of reference NMR spectra of the pure compounds and have been shown to be reproducible and accurate (15). Eight urine samples shown to have high levels of acetate, butyrate, and propionate were suspected to be contaminated with feces, and therefore were removed from statistical analysis.

Targeted Amino Acid Analysis of Serum

To prepare deproteinized extracts, 200 μL of serum samples were acidified with 50 μL sulfosalicylic acid. Intact proteins were removed after centrifugation and the supernatant from each sample was mixed with lithium diluent spiked with *S*-2-aminoethyl-L-cysteine. Samples were injected into an automated AAA (L-8900 Hitachi High-Technologies Corporation, Tokyo, Japan) equipped with ion-exchange chromatography using lithium citrate buffer. Each metabolite was detected spectrophotometrically after post-column reaction with ninhydrin reagent. Amino acid standards were intercalated with each sample and analyzed with a method developed by the Molecular Structure Facility (a part of UC Davis Proteome Core facility).

Hematology Measurements, Serum Biochemistry, and Hormone Assays

Samples of whole blood were collected for red blood cell (RBC) count, white blood cell (WBC) count, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin platelet counts (MCHC), plasma protein, neutrophils, monocytes (%), lymphocytes (%), eosinophils (%), basophils (%), platelets, plasma color, fibrinogen, erythrocyte morphology. Hemoglobin, hematocrit, WBCs, and RBCs were quantified with an automated electronic cell counter (Baker 9010 Analyzer; Serono-Baker, Allentown, PA). Hematological measurements and smear evaluations were performed at the CNRPC clinical laboratory with standard quality assurance procedures.

The standardized clinical biochemistry panel has been modified for use with rhesus monkeys and was performed at the UC Davis Veterinary Medical Teaching Hospital's clinical laboratory using an automated analyzer (Hitachi 917, Roche Biomedical, Indianapolis, IN) with standard quality assurance procedures. Measurements included sodium, potassium, chloride, total carbon dioxide (TCO2), anion gap, inorganic phosphorous, calcium, blood urea nitrogen (BUN), creatinine, glucose, total protein, albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine phosphokinase (CPK), alkaline phosphatase (ALK PHOS), gamma glutamyl transferase (GGT), lactate dehydrogenase (LDH), triglyceride, total cholesterol, and direct bilirubin.

Serum C-peptide, GIP, GLP-1, insulin, leptin, MCP-1, pancreatic polypeptide and PYY (total) were measured using a 96-well multiplex hormone magnetic bead panel that is specifically designed for non-human primates (Cat# NHPMHMAG-45, Milliplex Analyst, Millipore) according to the manufacturer's instructions.

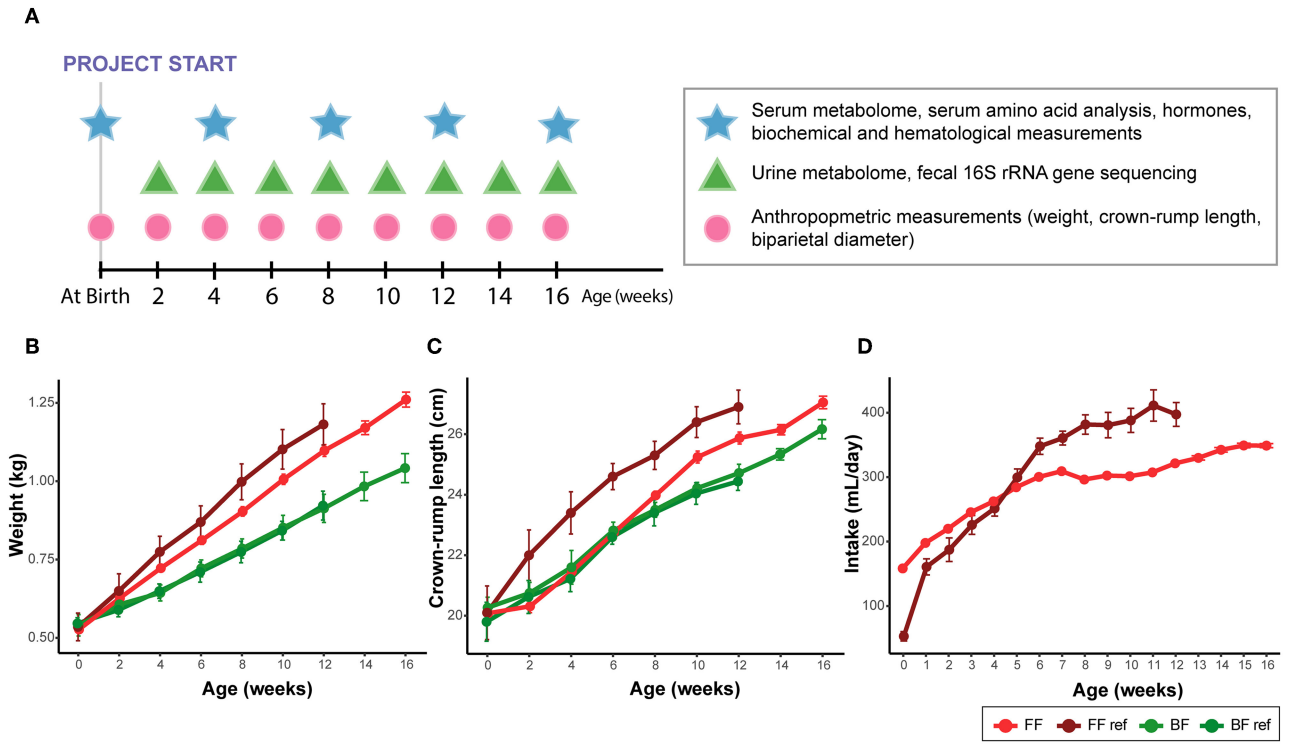

FIGURE 1 | (A) Summary of sample collection times. **(B)** Weight, **(C)** crown-rump length, and **(D)** formula intake of infant monkeys from birth to age of 16 weeks. Data were collected from breast-fed (BF) and pooled among all formula-fed (FF) rhesus infants from the current study and from the previous work [BF ref, FF ref (14)]. The amount of milk obtained from the exclusively breast-fed rhesus monkeys could not be recorded. Data are presented as mean ± SEM.

Fecal Microbial 16s rRNA Gene Sequencing

DNA was extracted from monkey stool samples collected at the second week and every 2 weeks thereafter until 16 weeks of age according to the protocol used in our previous rhesus monkey work (14). Briefly, fecal samples were washed with ice-cold PBS, followed by various of steps involving chemical lysis (Lysis buffer), heat treatment, physical lysis (bead beating), and use of QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA). Fecal DNA samples were amplified using the primer pair F515 and R806 against the Variable region 4 (V4) of bacterial 16S ribosomal RNA genes. Purified DNA libraries were submitted to the UC Davis Genome Center DNA Technologies Core for 250 bp paired-end sequencing on the Illumina Miseq platform. The Paired-end sequences were analyzed in Quantitative Insights Into Microbial Ecology (QIIME) pipeline v.1.9.0 (16). A closed-reference OTU picking procedure was used against the most current Greengenes core database ("gg_13_8_otus") (17). Differences in microbial community structures were explored using log-transformed weighted UniFrac distances followed by Principal Coordinate Analysis (PCoA). Differential abundance was evaluated using ANCOM (18) followed by FDR correction.

Statistical Analysis

To approximate normality, all data (weight, crown-rump length, biparietal diameter, metabolite concentrations) were log 10 transformed. Upon identification of significant variables, univariate statistical analyses were performed to confirm between-group differences. To evaluate the treatment effect across the entire dataset, both repeated measures ANOVAs (after removal of the measurements at birth, if measured) and repeated measures ANCOVAs (with cofactor as measurement at birth) were compared (*lme* function in nlme package, R). For each variable, repeated measures ANCOVA was used only in the situation that the effect of baseline (measurements at birth, if measured) was significant after model comparison using ANOVA.

To investigate the effect of protein content and addition of free amino acids on growth outcome, serum biochemistry, hormone, hematology, metabolome and fecal microbiome data, subsequent 3-way repeated measures ANOVA or ANCOVA were performed on all the FF groups with main effects as protein level, addition of free amino acid and time. The differences in month 1 were evaluated using data collected at both 2 and 4 weeks (urine data) and data at 4 weeks of age alone (serum data) using multiple independent *t*-tests or 2-way ANOVA follow by *post-hoc* Tukey. Pearson's product-moment correlation was used to compare the difference between circulating C-peptide, insulin and branched chain amino acids (BCAAs). The significance of correlation was evaluated using *t*-tests under the null hypothesis that the correlation was zero. FDR adjustment was used to correct for the multiple pairwise comparisons.

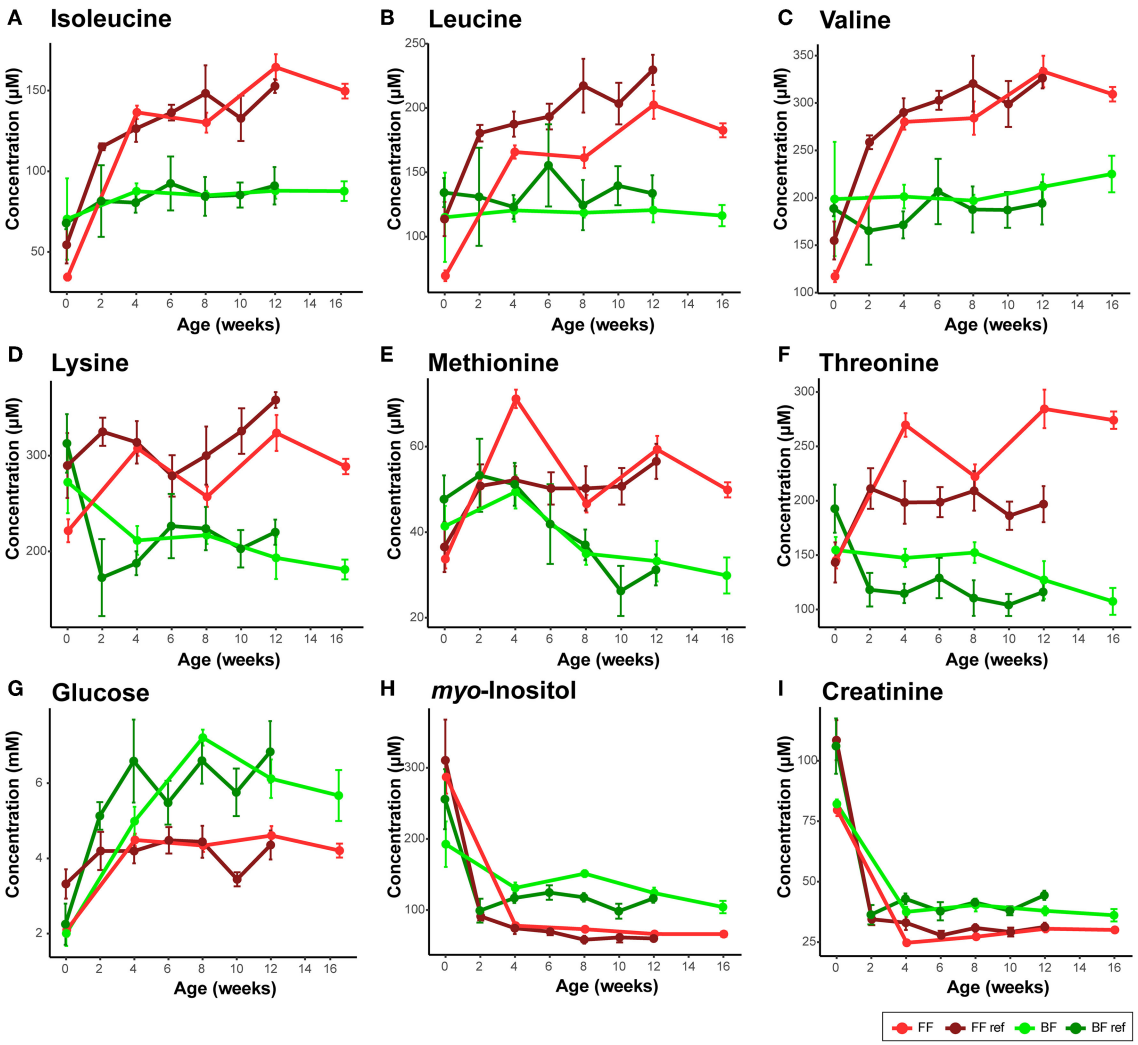

FIGURE 2 | Comparison of serum metabolites that are consistent between the breast-fed (BF) and formula-fed (FF) rhesus infants from the current study and the breast-fed reference (BF ref) and formula-fed reference (FF ref) from previous work (14). Serum metabolites including **(A)** isoleucine, **(B)** leucine, **(C)** valine, **(D)** lysine, **(E)** methionine, **(F)** threonine, **(G)** glucose, **(H)** *myo*-inositol, **(I)** creatinine were measured from birth to 16 weeks of age in the current study. Data are presented as mean ± SEM.

HOMA-IR was calculated as the product of glucose (mmol/L) × insulin (mIU/L) /22.5. QUICKI was calculated as the product of 1/[log(I) + log(G)] where I is insulin (μU/mL) and G is fasting glucose (mg/dL).

RESULTS

Rhesus Infants Consuming a Formula With a Protein Content Slightly Lower Than Rhesus Milk Still Revealed Higher Weight Gain, Insulin, and Elevated Circulating Amino Acids

We previously reported that when feeding a formula with higher protein content than rhesus milk, FF infants had an accelerated growth trajectory in comparison to their BF counterparts (14). In the present study, when feeding formulas with a slightly lower protein content than that of rhesus milk, all the FF infants exhibited moderate weight gain compared with the FF group from our previous work, but still showed faster weight gain compared with their BF counterparts (**Figure 1B**, pairwise comparison against all formula-fed groups, repeated measures ANCOVA, all individual $p < 0.03$); however, the differences in crown-rump length (**Figure 1C**) and biparietal diameter were not significant. The difference observed in the present study compared to the FF group from our previous work may be due to a lower intake of formula (**Figure 1D**). Nutrient composition of the diets is presented in **SI Table 1**.

In human infants, we and others have reported higher circulating nitrogenous waste products (urea/BUN) in FF compared to BF infants (19–22). Our previous work on infant rhesus macques did not find a significant difference in serum

and urinary urea between infants who were breast-fed and those fed infant formula designed for human infants (14), which may be due to a higher dietary protein requirement of rhesus infants than human infants (23). In the present study, as formula protein was reduced to slightly lower than typically observed in rhesus milk, we observed significantly lower serum urea and ammonia in infants consuming formula compared to their BF counterparts (Repeated measures ANCOVA, **SI Figure 1**). Serum creatinine, another nitrogenous compound that is not different between BF and FF human infants, was consistently lower in the FF rhesus infants (**Figure 2I**, confirmed using both ^{1}H NMR-based metabolomics analysis and clinical biochemical assays, **SI Figure 2**). We further quantified the urinary metabolites and observed a significantly lower creatinine level in these FF infants, which was not observed in our previous rhesus infant study (**SI Figure 3**).

Through assessment of a panel of clinical biomarkers, measurements of ALT, AST, and GGT that were previously reported to be higher in human BF infants (22) were either not different or higher levels were found in FF rhesus infants (summarized in **Table 1**, **SI Figure 5**). A panel of biochemical and hematological measurements revealed that serum albumin, a marker of undernutrition, was significantly lower in these FF rhesus infants. This lower level of albumin was coupled with lowered anion gap in these FF infants (**SI Figure 5**). Significantly lower hemoglobin, hematocrit and MCHC values were also observed (**SI Figure 7**), but were not significantly different from BF infants in our previous monkey study (14).

Importantly, through ^{1}H NMR-based metabolomics analysis, we found that when feeding the formula containing a level of protein approaching the minimum protein requirement of developing rhesus infants, circulating levels of BCAAs (leucine, isoleucine, and valine), lysine, methionine, threonine and asparagine reported to be higher in FF human infants and FF rhesus infants (from our previous work) were still significantly higher in the rhesus infants fed the current study formulas (**Figure 2**, **Table 2**), suggesting that reducing protein content in formula alone cannot completely reverse the formula feeding-specific metabolic phenotype on serum amino acid levels. Higher levels of circulating serine and phenylalanine were found in our previous study on FF rhesus infants but were not significantly different in the current study (**SI Figure 13**). Targeted amino acid analysis, performed in parallel with NMR-based metabolomics analysis, revealed similar results (summarized in **Table 2**).

Serum insulin was elevated in rhesus infants fed formula with a protein level higher than rhesus milk (14), and was still significantly higher in FF rhesus monkeys fed the current formula (repeated measures ANCOVA, **Figure 3A**). Serum C-peptide was also significantly higher in these FF infants and correlated well with serum insulin levels (repeated measures ANCOVA, **Figures 3B,C**). The higher levels of serum insulin and C-peptide are strongly correlated with higher serum isoleucine, leucine, valine, methionine, and threonine (Pearson correlation $r > 0.4$, $p < 0.001$ after FDR adjustment), supporting the regulatory role of these amino acids on insulin and C-peptide secretion (24). We have previously observed this positive relationship between BCAAs and insulin in human infants, suggesting this observation is translational to human infants (21).

Some breast feeding-specific markers were consistently altered. In agreement with observations in BF human infants, BF rhesus infants also exhibited higher levels of circulating *myo*-inositol, succinate, fumarate, and ketone bodies (summarized in **Table 2**). Circulating glucose and pyruvate were consistently higher in BF rhesus infants, but were not observed to be different between BF and FF human infants (**Figure 2**, **Table 2**). The differences in glucose were confirmed using clinical biochemical assays (**SI Figure 6**).

Reducing the Protein Content of Infant Formula Suppresses Intake, Lowers Weight Gain, and Improves the Formula-Fed Specific Metabolic Phenotype

To determine the metabolic impact of feeding a reduced protein formula, differences between infants fed regular and reduced protein formula were evaluated. Overall, the reduced protein formula had a protein composition identical to regular formula, but 14.3% less of each amino acid. Correspondingly, rhesus infants receiving the reduced protein formula showed 10–20% lower protein intake throughout the study compared to those fed regular formula (**SI Figure 14**). The lower protein content moderately decreased overall formula intake ($p = 0.03$, repeated measures ANCOVA), and this effect on appetite was most profound in the first month. Correspondingly, feeding the reduced protein formula significantly decreased weight gain measured at 2 and 4 weeks of age ($p < 0.05$, 3-way ANCOVA), but not at later time points (**Figure 4**).

In parallel with the changes in formula intake and weight gain, at 4 weeks of age lower levels of circulating insulin and C-peptide as well as lower insulin resistance (homeostasis model assessment for insulin resistance; HOMA-IR) and higher insulin sensitivity (quantitative insulin sensitivity check index; QUICKI) were observed in the monkeys consuming reduced protein formulas (**Figures 3**, **5**). These improvements in glucose metabolism were further coupled with a significant reduction of circulating BCAAs at all time points measured (tested on both 3-way ANCOVA and ANOVA model, $p < 0.05$ for both NMR and AAA data, **SI Figure 8**). However, other serum markers specific for formula-feeding including methionine, threonine, asparagine, and lysine were not significantly reduced by lowering the protein level in formula (**SI Figures 9**, **10**). At 4 weeks of age, indicators of collagen breakdown (hydroxylysine, hydroxyproline, and glycine), serine (precursor of glycine), homocysteine, and ethanolamine were significantly higher in serum from infants consuming the reduced protein formula (2-way ANOVA, $p < 0.05$ after FDR correction, **SI Figures 15A–F**). A trend toward higher aspartate was also observed in the infants consuming reduced protein formula (2-way ANOVA, $p < 0.05$ without FDR correction, **SI Figure 15G**).

As expected, reducing the protein level in infant formula led to a significant change in urinary metabolites, particularly in the first month when physiological and hormonal changes were most profound. In comparison to infants consuming

TABLE 1 | Metabolic hormones, biochemical measurements, hematological measurements.

Class	Variables	BF vs. FF	Regular vs. reduced protein formula	With vs. without adding amino acids	Graphical illustration
Metabolic hormones	C-peptide	FF ($p < 0.001$)	ND	ND	**Figure 3**
	GIP	ND			
	GLP-1				
	Insulin	FF ($p < 0.001$)			**Figure 3**
	MCP-1	ND			
	Pancreatic polypeptide			Without adding AAs ($P < 0.001$)	**SI Figure 4**
	PYY (total)			ND	
Clinical biochemistry	Sodium (mM/L)				
	Potassium (mM/L)				
	Chloride (mM/L)	BF (#)			**SI Figure 5**
	Calcium (mg/dL)	ND			
	Phosphorous (mg/dL)				
	TCO2 (mM/L)	FF ($p < 0.001$)			**SI Figure 5**
	Anion gap	BF ($p < 0.001$)			**SI Figure 5**
	BUN (mg/dL)	BF ($p = 0.02$)	Regular formula (#)		**SI Figure 1**
	Creatinine (mg/dL)	BF ($p < 0.001$)	ND		**SI Figure 2**
	Glucose (mg/dL)	BF ($p < 0.001$)			**SI Figure 6**
	Total protein (g/dL)	ND			
	Albumin (g/dL)	BF ($p = 0.005$)			**SI Figure 5**
	ALT (U/L)	FF (#)			**SI Figure 5**
	AST (U/L)	FF ($p = 0.003$)			**SI Figure 5**
	CPK (U/L)	ND			
	ALK PHOS (U/L)	FF ($p = -0.02$)		With adding AAs (#)	**SI Figure 5**
	GGT (U/L)	ND		ND	
	LDH (U/L)				
	Cholesterol (mg/dL)				
	Triglyceride (mg/dL)				
	BILI Total (mg/dL)				
Hematology	WBC (×10^3/μL)				
	RBC (×10^6/μL)				
	Hemoglobin (gm/dL)	BF ($p < 0.001$)	Regular formula ($P < 0.001$)		**SI Figure 7**
	Hematocrit (%)	BF ($p > 0.001$)	Regular formula ($P < 0.001$)		**SI Figure 7**
	MCV (fL)	ND	ND		
	MCH (pg)				
	MCHC (pg/fL)	BF (#)		With adding AAs ($P < 0.001$)	**SI Figure 7**
	Platelets (×10^5/μL)	ND		ND	
	Plasma protein (gm/dL)				
	Total WBC (×10^3/μL)				
	SEG Neutrophils (%)				
	Monocytes (%)				
	Lymphocytes (%)				
	Eosinophils (%)				
	Basophils (%)				
	Platelets				
	Plasma color				
	Erythrocyte morphology				

FF, higher in the formula-fed group; BF, higher in the breast-fed group; Regular formula, higher in the regular formula than reduced protein formula; With adding AAs, higher in formula groups with addition of free amino acids; Without adding AAs, higher in formula groups without addition of free AAs.
Significant difference was evaluated independently using repeated measures ANCOVA at $p < 0.05$ after FDR correction. (#) $p < 0.05$ before FDR correction. ND, not significantly different.

TABLE 2 | Breast and formula feeding- specific metabolite markers in serum.

Class	Serum metabolites	Human infant reference [a]	Human infant reference [b]	Rhesus infant reference [c]	Current study	Confirmed using amino acid analyzer	Graphical illustration
Essential amino acids	Leucine	FF	FF	FF	FF	Yes	**SI Figure 8**
	Isoleucine	FF	FF	FF	FF	Yes	**SI Figure 8**
	Valine	FF	FF	FF	FF	Yes	**SI Figure 8**
	Lysine	FF	FF	FF	FF	Yes	**SI Figure 9**
	Phenylalanine			FF	ND	Yes	
	Methionine	FF	FF	FF	FF	Yes	**SI Figure 9**
	Threonine	FF	FF	FF	FF	Yes	**SI Figure 9**
	Tryptophan				FF	No*	
	Histidine				ND	Yes	
Non-essential amino acids	Alanine			FF	FF	Yes	**SI Figure 10**
	Arginine			FF	ND	Yes	
	Asparagine		FF	FF	FF	Yes	**SI Figure 10**
	Aspartate			FF	ND	Yes	
	Glutamate		BF	FF	ND	Yes	
	Glutamine			BF	ND	Yes	
	Serine		BF	FF	ND	Yes	
	Taurine			FF	ND	Yes	
	Tyrosine	FF	FF		ND	Yes	
	Proline	FF			ND	Yes	
	Creatine	FF	FF		ND	Yes	
	Glycin				ND	Yes	
	Ornithine				ND	Yes	
Amino acid derivatives	Hydroxyproline				BF	Yes	**SI Figure 10**
	Dimethylamine				BF		
	Creatinine			BF	BF		**SI Figure 2**
Sugars	Glucose			BF	BF		**SI Figure 6**
	Galactose			FF	FF		**SI Figure 6**
	myo-inositol		BF	BF	BF		**SI Figure 6**
Energy metabolism	Pyruvate			BF	BF		**SI Figure 11**
	Citrate		BF	BF	ND		
	Succinate		BF	BF	BF		**SI Figure 11**
	Fumarate		BF	BF	BF		**SI Figure 11**
	Lactate				BF		**SI Figure 11**
	Malate				BF		**SI Figure 11**
	Acetylcarnitine		BF		BF		
Ketones	3-hydroxybutyrate		BF		BF		**SI Figure 12**
	Acetoacetate		BF	BF	BF		**SI Figure 12**
Others	Allantoin			FF	ND		
	Urea	FF	FF	ND	FF		**SI Figure 1**

Significant difference was evaluated independently using repeated measures 2-way ANOVA or ANCOVA at $p < 0.05$ after FDR correction. FF, higher in the formula-fed group; BF, higher in the breast-fed group; ND, no significant difference.

[a] Data from NMR metabolomics work extracted from a human study at approximately 180 min post-meal (21).

[b] Data from NMR metabolomics work extracted from a human study at approximately 165 min post-meal with either no or little complementary food consumed (20).

*[c] Data extracted from our previous rhesus monkey study (14). Formula composition from this study is also summarized in **SI Table 1**.*

[a–c] Sample preparation, spectral acquisition and compound quantification followed the same protocol as the current study.

**In the current study, serum tryptophan was significantly higher in the FF group. However, the protein precipitation method used in preparation of AAA released higher levels of protein-bound tryptophan compared to the ultrafiltration method used in the NMR sample preparation that captured only free tryptophan. The trend is in agreement with our previous work that also reported on samples extracted using protein precipitation or ultrafiltration (14).*

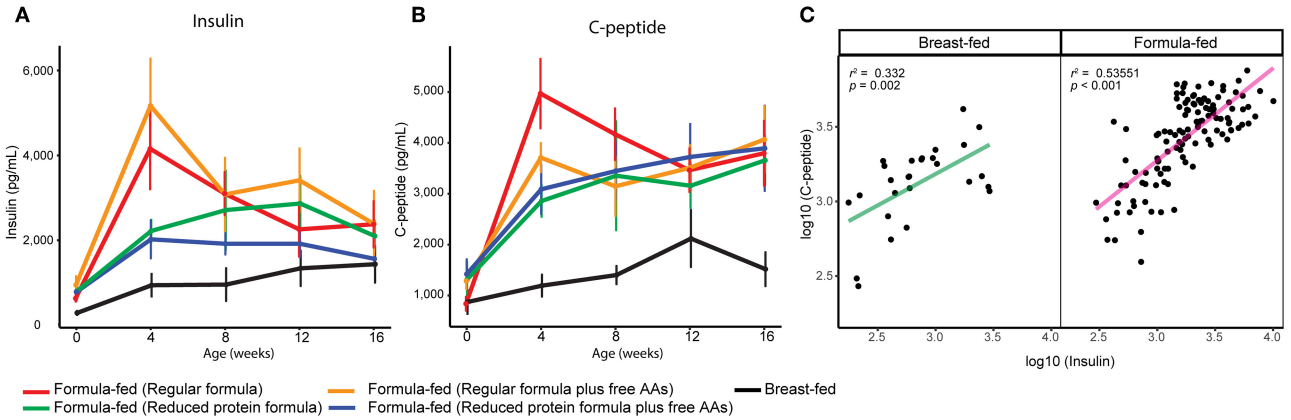

FIGURE 3 | Serum **(A)** insulin and **(B)** C-peptide of breast-fed and formula-fed rhesus infants from birth to 16 weeks of age. Data are presented as mean ± SEM. **(C)** Scatter plot demonstrates a positive correlation between serum measurements of insulin and c-peptide.

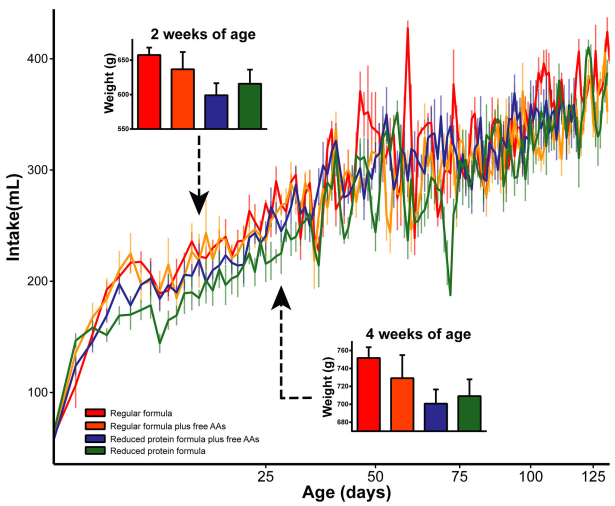

FIGURE 4 | Daily intake was significantly increased by increasing the amount of protein in infant formula. As a result, weights at 2 and 4 weeks of age were significantly increased by the elevated protein content in infant formula. Data are presented as mean ± SEM.

regular formula, those consuming the reduced protein formula exhibited lower levels of urinary amino acids (both essential and non-essential), intermediate products of amino acid metabolism, nitrogenous waste products, and products from microbial degradation of protein or host-microbe co-metabolism (2-way ANOVAs, $p < 0.05$ after FDR correction, **SI Figure 16**).

Adding Free Amino Acids in Infant Formula Did Not Alter Milk Intake, but Did Result in Several Metabolic Changes

Addition of free amino acids (such as glutamate) to infant formula has been reported to promote satiation and satiety as well as reduce total intake volume (12). To evaluate the potential role of free amino acids on appetite regulation using the infant rhesus macaque model, four free amino acids present in relatively high concentration in human breast milk (glutamate, glutamine, alanine, and taurine) were added to the formula. We failed to observe an effect of free amino acids on intake and weight gain. Among the four free amino acids added to the formula (glutamate, glutamine, alanine, and taurine), only taurine was observed to be significantly higher in the urine (repeated measures ANOVA, $p < 0.05$ after FDR correction, **SI Figure 17**). This is expected since unlike most amino acids that have renal reabsorption rates of 98–99%, renal reabsorption of taurine depends on taurine intake, and ranges from 40 to 99.5% (25). Interestingly, for the rhesus infants who consumed formula with added free amino acids, significantly lower levels of pancreatic polypeptide ($p < 0.05$ after FDR correction, **SI Figure 4**) and a trend toward higher circulating levels of ALK phosphate ($p < 0.05$ before FDR, **SI Figure 5**) were observed. Addition of free amino acids to formula resulted in a trend toward lower serum levels of betaine (**SI Figure 17**). Furthermore, several amino acids and nitrogen-containing metabolites were significantly higher in the urine of those receiving free amino acids in their formula (repeated measures ANOVA, $p < 0.05$ after FDR correction, **SI Figure 17**).

An Additive Effect of Free Amino Acids With the Base Formula Was Observed

Compared to the infants consuming the reduced protein formula, those consuming the reduced protein formula with added free amino acids revealed the lowest circulating BCAA level (**SI Figure 8**) and lowest urinary 3-hydroxyisovalerate (byproduct of BCAA degradation, **SI Figure 16**) in the first month of age, approaching the level observed in the BF reference group.

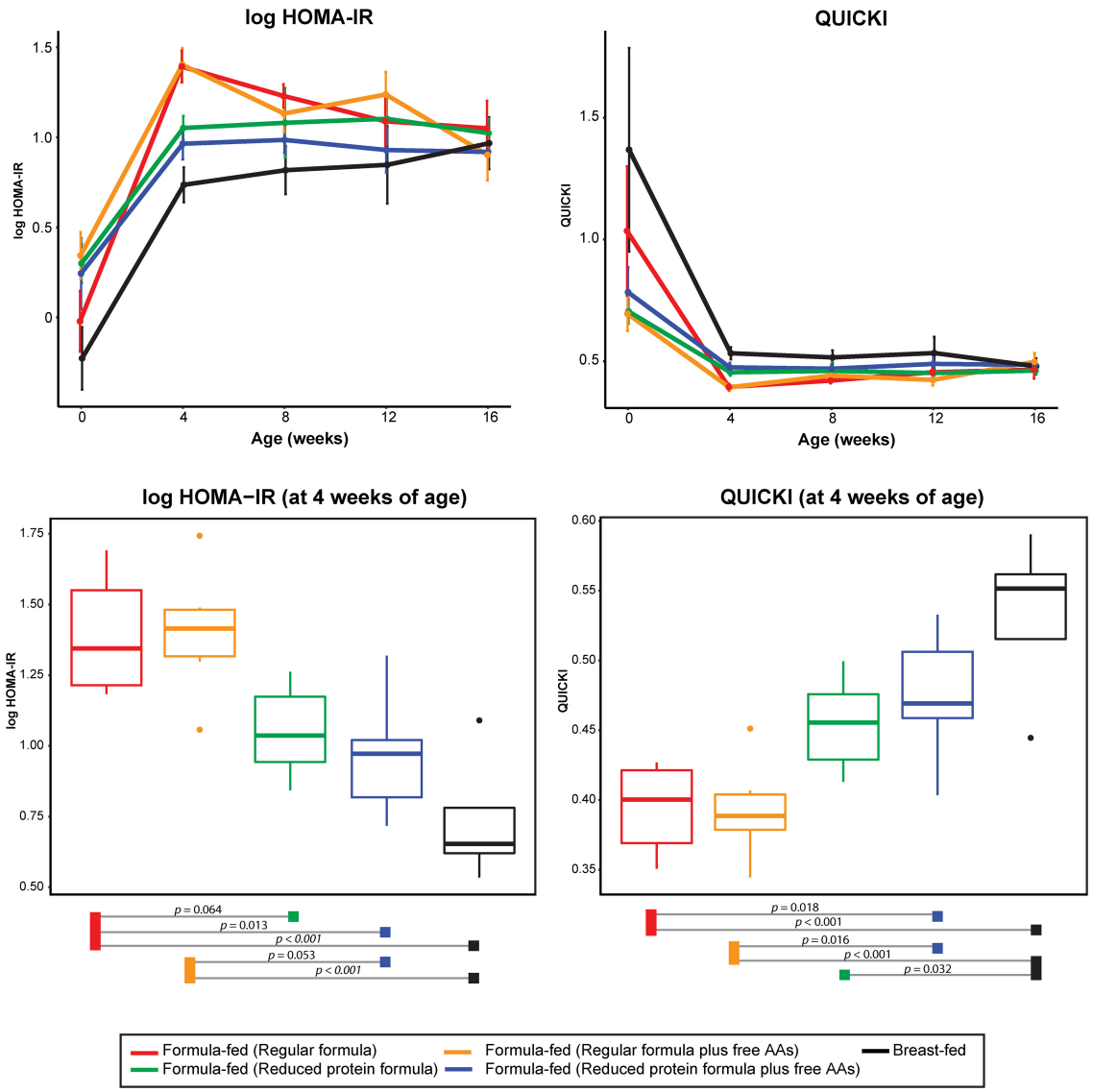

FIGURE 5 | Change of log transformed HOMA-IR and QUICKI level over time and at 4 weeks of age. The statistical difference at 4 weeks of age was evaluated using ANOVA follow by *post-hoc* Tukey. Data are presented as mean ± SEM.

Reducing Protein Content in Formula Alters Fecal Microbial Composition in an Intake-Dependent Manner

To evaluate the impact on gut microbial composition, fecal samples collected from the second week onward were examined using 16s rRNA gene sequence analysis. The overall community profile of both BF and FF rhesus infants included the dominant phylum-level representatives from Firmicutes and Actinobacteria, followed by Bacteroides, with the most prevalent bacterial genera identified as *Lactobacillus*, *Bifidobacteria* and *Prevotella* (**SI Figure 18**). Differences in fecal microbiota depending on feeding mode (breast or formula) were observed (significantly different OTUs at the genus level are summarized in **SI Table 2**, evaluated using ANCOM, $p < 0.05$ after FDR correction). However, the fecal microbiome profile of BF rhesus infants is different from that of BF human infants, who tend to be dominated by *Bifidobacteria*. Therefore, only the bacterial community profiles of FF rhesus infants were compared across all time points. A more distinct difference in fecal microbial profile between infants fed regular formula and those fed reduced protein formula was observed in the first month of age (2 and 4 weeks) which was not obvious in the later months (**SI Figure 19**). This difference is, in part, due to a significantly higher level of fecal *Bifidobacterium* and lower levels of fecal *Dorea* and *Ruminococcus* (from Ruminococcaceae family) in the stools from the reduced protein formula group compared with stools from the regular formula group (**SI Figure 20**, evaluated using ANCOM, $p < 0.05$ after FDR correction). Fecal samples

obtained from monkeys consuming the regular formula had higher diversity than those consuming the reduced protein formula at 4 weeks of age. This observation was not observed in the later weeks ($p < 0.05$, one-way ANOVA follow by *post-hoc* Tukey HSD test). There was no consistent alteration of specific microbial taxa due to free amino acid supplementation in infant formula, suggesting that the added free amino acids may be rapidly absorbed in the upper gastrointestinal tract.

DISCUSSION

Infant formula is the closest alternative to human milk when breastfeeding is not feasible or desired. The general goal has been to improve the formulation by matching its nutrient content to human milk as closely as possible. However, infant formula largely lacks free amino acids, bioactive and functional proteins, and is formulated with a higher content of protein than human milk. It has been assumed that when energy intake is adequate, protein is utilized for maintaining the body amino acid pool and is deposited in tissue instead of being used as an energy source. Infant formula with more protein beyond what is required does not provide an advantage to infants, as the high levels of circulating amino acids may put an additional burden on the liver and the renal system to metabolize and excrete the excess nitrogen. When optimizing infant formulas, the primary goal should not be to focus on making it similar to human milk, but to make the performance of FF infants similar to that of BF infants. A system-wide omics approach represents a powerful tool for monitoring metabolic consequences and gut microbial profiles in response to early diet beyond what can be captured using clinical growth outcome measurements, and further provides insight for development of improved infant formulas.

In the present study, we validated that the infant rhesus monkey is a highly translational and robust preclinical model to understand the metabolic differences between BF and FF human infants. In comparison to the BF group, all FF groups (regardless of the type of formula) exhibited accelerated weight gain in combination with distinct differences in fecal microbiota composition, as well as serum and urine metabolic profiles. Reducing the protein content in infant formula substantially reduced formula intake and weight gain, as well as serum BCAAs levels, but did not alter other circulating amino acids, such as methionine, threonine, asparagine, and lysine, which were also higher in FF infants compared with BF infants. In addition, through testing study formulas with protein levels slightly lower than rhesus milk, we observed that these FF rhesus infants showed reduced protein catabolism that can be characterized as lowered circulating levels of urea, ammonia, albumin and creatinine as well as lower excretion of creatinine in urine. However, despite having low protein levels in the study formulas, the typical formula-fed phenotype that includes high circulating insulin and amino acids was not improved. Our results suggest that while the total protein in formula is an important factor that could be modified to improve physiological and metabolic profiles of the FF infant, it is not the only factor that contributes to the FF phenotype. We speculate that a dietary component (other than protein alone) may have a role in maintaining the high levels of insulin and C-peptide in these FF infants. Further studies are needed to trace the downstream amino acid catabolic by-products to determine whether BCAA clearance is sub-optimal.

Reducing the protein level in infant formula revealed that the largest physiological, metabolic and fecal microbial differences occur before and at the first month of age (equivalent to approximately 3 months of age in humans). We expect that protein/amino acids from the diet, if utilized efficiently, should largely be absorbed before moving past the terminal ileum. Different microbial profiles obtained from infants consuming regular or reduced protein formulas were observed primarily during the first month of age. These results suggest that, at least during early infancy, high levels of protein may exceed absorption capacity, and reach the colon to become a source of nutrients and influence the abundance of various gut microbes. We observed that as the protein level in infant formula was reduced, levels of fecal microbes (*Bifidobacterium* and *Ruminococcus* from the Ruminococcaceae family) that are known to have varying ability to utilize complex carbohydrates (26) increased (**SI Figure 20**). After the first month of age, the response toward different protein content in the formula became less pronounced. This time-dependent change paralleled the difference in intake that was most profound in very early age.

Current views suggest that BF infants are born with an innate ability to regulate food intake in response to internal cues of appetite, regardless of mother's milk supply (27). In contrast, bottle-feeding has been proposed to promote more parental control and less self-regulation than breast-feeding (28). This conclusion is partially due to the fact that FF infants have been shown to consume significantly greater volumes of milk than BF infants (8, 9). Regardless, overfeeding of FF infants (who have an associated higher consumption of protein and energy) is associated with increased growth rate and adiposity in early life (29). Early growth acceleration during infancy has been associated with an increased odds of becoming overweight or obese in adult life (30–32). Thus, regulating intake is one key aspect for preventing early growth acceleration.

It is thought that infants have an innate ability to adjust their intake based on the energy density of their food (33); therefore, an appropriate protein: fat ratio may be key to preventing accelerated weight gain during infancy. Several clinical observations have also suggested that the nutrient composition of the diet may also influence how infants regulate their intake. For example, in a parent-blinded, randomized cohort, Ventura *et al.* observed a significant reduction in feeding volume and meal duration when comparing provision of a formula containing free glutamate to an isocaloric standard formula (12, 34). Formula with low protein quality (i.e., with inadequate essential amino acids) may promote higher consumption, as it was observed that infants increased their consumption when being fed a casein-predominant formula regardless of whether the protein:energy ratio was low (35) or high (36). However, when examining formula with a more balanced amino acid profile (whey-casein ratio: 60:40, 2.2 g protein/100 kcal), the differences in daily consumption and energy were not significant when infants were fed a reduced

protein formula (70:30, 1.8 g protein/100 kcal) after adjusting for multiple correction, sex, smoke exposure and maternal education (37).

Use of rhesus monkeys as a model provides direct information on consumption since the amount of formula consumed is responsive to infant cues of hunger and satiety and there is no parental encouragement. By feeding isocaloric formulas, we demonstrated that, in early life, higher protein in infant formula induces greater appetite and greater calorie intake, suggesting a lack of ability to completely self-regulate intake based on meal energy density alone. There is a possibility that the early development of the brain reward system toward food intake is incomplete, and the combination of greater energy and protein intake that leads to attenuated circulating BCAAs, insulin, C-peptide, and accelerated growth may preprogram long-term changes. We speculate that a formula with high protein content may diminish the response to internal cues of satiety. However, this finding is only significant in a time sensitive window and needs to be carefully investigated in a clinical study prior to the introduction of complementary food that displaces the intake of breast milk or formula. In this study, we failed to observe a significant effect of free amino acids on regulating formula intake using the rhesus monkey model. However, pancreatic polypeptide, a gut hormone that was previously found to reduce appetite and food intake in humans (38), was lower in the FF groups with additional free amino acids (**SI Figure 4**).

One potential limitation of the study is that only female rhesus infants were used in order to reduce the within group variations induced by different sexes. In humans, female infants have been shown to have less appetite, are slightly less responsive to cues of feeding and are more sensitive to internal cues of satiety compared to their male counterparts (39). In addition, the formulas used in the current study had a lower protein level (protein accounts for 8.4% energy in the regular formula, and 7.2% in the reduced protein formula) in comparison to the high protein formula used in the European Childhood Obesity Trial [protein provided 11.7% energy in their high protein formula, and 7.1% energy in the low protein formula (40)]. Furthermore, the formulas used in that study were casein-predominant, in which tryptophan is the limiting amino acid; phenylalanine and tyrosine are high in casein. In comparison to the high protein formula, infants who consumed the low protein formula showed lower levels of circulating phenylalanine and tyrosine but these amino acids were still significantly higher than in the BF reference group. In contrast, infants who consumed the low protein formula showed a significantly lower circulating tryptophan level in comparison to the BF reference group (40). In the present study, since whey-predominant formulas were fed, circulating phenylalanine and tyrosine were not different between BF and FF infants, nor were they influenced by the reduced protein level in formula. Additionally, circulating tryptophan was higher in FF compared to BF infants, and was not influenced by the level of protein in the formula.

It is known that mature rhesus milk is considerably higher in protein (15–20 g/L) than human milk (8–9 g/L) (23) and contains significantly less free amino acids (13). This may lead to differences in dietary protein and free amino acids requirement between human and rhesus infants. Human infants may be less tolerant and more metabolically sensitive to high levels of protein in formula. An infant formula designed for human infants may provide a protein level that is considerably high for human infants but approach the lowest level that is acceptable for rhesus infants. Yet, the rhesus macaque is still a valid research model to evaluate the phenotypic and metabolic gap between breast milk and any given formula. The lesson learned from the comparison between formula groups is robust and can serve as a starting point to unveil the biological mechanism behind the "early protein hypothesis."

CONCLUSION

Our data on a preclinical rhesus infant model concludes that protein in formula is an important factor that can be modified to improve the physiological and metabolic outcomes of FF infants. However, although reducing the protein and adding free amino acids to formula is one step forward, it still insufficient to reverse the FF-specific accelerated growth, and BCAA-induced high insulin phenotype. Further research is warranted to explore other dietary factors that may be responsible for inducing the systematic metabolic manifestation that occurs with formula-feeding.

DATA AVAILABILITY STATEMENT

The 16s sequencing data is available from European Nucleotide Archive (accession code ERP117320) and Qiita (study ID 12033). The raw data supporting the conclusions of this article will be made available from the corresponding author on request, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by University of California, Davis, Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

CS had full access to all of the data in the study and takes responsibility for the integrity of the data and accuracy of the data analysis. Study concept and design: BL and CR. Metabolomics analysis: XH and JS-O. Microbiome analysis and statistical analyses: XH. Interpretation of data and drafting of manuscript: XH and CS. Obtained funding: BL, CR, and CS. Editing of manuscript: all authors. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved the final version for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2019.00563/full#supplementary-material

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Health Service Use and Treatment Choices for Pediatric Eosinophilic Esophagitis: Findings From a Cross-Sectional Survey of Australian Carers

Nicole Hannan[1], Amie Steel[2], Sara S. McMillan[1] and Evelin Tiralongo[1]*

[1] School of Pharmacy and Pharmacology, Griffith University, Southport, QLD, Australia, [2] Faculty of Health, Australian Research Centre in Complementary and Integrative Medicine, University of Technology Sydney, Ultimo, NSW, Australia

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***Correspondence:**
Nicole Hannan
nicole.hannan2@griffithuni.edu.au

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Objectives: The incidence and the prevalence of eosinophilic esophagitis (EoE) are increasing, and healthcare utilization among children with EoE is high. This study provides novel insights into the health services and the treatments, including complementary medicines (CMs), used by carers to manage their children's EoE as well as the carers' beliefs and attitudes toward these treatments.

Methods: A national cross-sectional online survey was conducted in Australia between September 2018 and February 2019. The survey included questions about health service and treatment utilization, health insurance and government support, health-related quality of life of children with EoE and their carers, views and attitudes toward CM use, and perceived efficacy of treatment.

Results: The survey was completed by 181 carers (96.6% of whom were mothers) of EoE children. Most children (91.2%, $n = 165$) had seen a medical doctor for their EoE, and almost half had consulted with a CM practitioner (40.3%, $n = 73$). Pharmaceuticals ($n = 156$, 86.2%) were the most commonly used treatment option, followed by dietary changes ($n = 142$, 78.5%), CM products ($n = 109$, 60.2%), and CM therapies ($n = 42$, 23.2%). Most children received care from numerous practitioners on multiple occasions, indicating a substantial financial and treatment-related burden.

Conclusions: A variety of practitioners are involved in the care of children with EoE, and a high rate of CM use warrants further attention to ensure that appropriate treatment is provided. Carer involvement and guidance, combined with individual practitioner expertise, referrals, and collaboration between providers, is essential to successfully navigate this complex disease and provide adequate care for these patients.

Keywords: complementary medicine, therapy, allergy, child, EoE

INTRODUCTION

Eosinophilic esophagitis (EoE) is a rare antigen-driven inflammatory gastrointestinal disorder characterized by elevated levels of eosinophils in the esophagus, esophageal dysfunction, and gastrointestinal symptoms (1). EoE incidence is increasing globally, with an estimated prevalence of one in every 2,000 people (2–4). International clinical guidelines list EoE first-line treatment options as proton-pump inhibitors, swallowed topical steroids, elimination diets, and elemental formula (5–8). While there are no national guidelines in Australia for the management of EoE, international guidelines are usually applied (9).

Given the high frequency of healthcare utilization among children with EoE (10), it is important to better understand the patterns of use in order to improve the support for these children and their carers as well as facilitate more coordinated and collaborative care between healthcare providers. Research suggests that complementary medicine (CM), a diverse range of medical and healthcare practices and products not currently regarded as part of conventional medicine (11), may be included in the range of healthcare accessed by carers of children diagnosed with gastroenterological conditions in Australia (12) and abroad (13). In order to address EoE symptoms, carers may choose CMs for their children, under the assumption that CMs are safe (14, 15); however, to our knowledge, no research has examined all healthcare accessed for pediatric EoE, including CM. In response, this study is the first to describe the health services and the treatments, including CMs, used by carers to manage their children's EoE as well as the carers' beliefs and attitudes toward these treatments.

METHODS

Definitions

CM involves two broad classifications, defined in this study as either CM products (i.e., probiotics) or CM therapies (i.e., massage) (11).

Study Design and Setting

A national cross-sectional online survey was conducted between September 2018 and February 2019. Ethics approval was obtained from the Griffith University Human Research Ethics Committee (#2018/120). The survey included the following domains: demographics; health service and treatment utilization; health insurance, government support, and rebates; health-related quality of life (HRQoL) of children with EoE and their carers; views and attitudes toward CM use; and perceived efficacy of treatment.

Survey Design

The survey instrument was designed to take 20–30 min to complete and incorporated pre-existing validated tools, namely, Bakas Caregiving Outcomes Scale© (16), PedsQL™ Eosinophilic Esophagitis Module Standard Version 3.0 Parent Reports© (17), and PedsQL™ Infant Scales© (18), along with other adapted survey items (see **Table 1**). In addition to the pre-existing instruments, the survey items were drafted to confirm eligibility [mandatory questions included: "Did your child have an endoscopy to assist with EoE diagnosis?" and "Has your child been diagnosed with EoE by a pediatric gastroenterologist (or other medical specialist)?"], ensure that the questions addressed the CM use in children, not the carer, and gauge treatment burden [the workload attributed to healthcare, and its impact on patient well-being and functioning (38)], and access to funding support for EoE patients. This included questions about access to private health insurance and a government-issued healthcare card and/or carer allowance. Australia's public health system provides access to a wide range of hospital and health services for all Australians at low or no cost (39). In Australia, additional private health insurance can be purchased to cover specific costs related to private hospital treatment and other medical services (40). Carer allowance is means tested and is available for those persons who provide additional daily care to a child who has a serious chronic illness (41). Healthcare cards can reduce the cost of certain prescription medications and medical doctor consultations and are issued to persons receiving various government payments or subsidies, including carer allowance (42).

The survey was tested for content and face validity, with feedback obtained by two parents of children with chronic disease using a paper version of the survey, followed by online testing *via* the Survey Gizmo® platform by the parent of a child with eosinophilic gastroenteritis. The lead researcher and the parent of a child with EoE also tested the online version on different devices (e.g., tablet, phone, and laptop). Minor changes to improve readability and understanding were made based on the feedback from the different parties and following discussions among the research team.

Participants

The study participants were English-speaking carers of children with a confirmed EoE diagnosis ($\leq$18 years of age) in Australia. The target survey sample size of 210 parents of EoE children was determined to achieve a 95% confidence level, confidence interval of 5, and population of 462 from a prevalence rate of 1 in 10,000 (2, 43).

Recruitment

Purposive convenience and snowball sampling were employed. The responses were limited to one survey per family; if more than one child in the family had EoE, the respondents were asked to complete the survey for the eldest child only.

The Australian pediatric EoE support network, AusEE Inc., promoted the survey to their network of consumer members, their medical advisory board, other specialist doctors, and organizations such as Allergy and Anaphylaxis Australia and Allergy and Immunology Foundation of Australasia. Professional associations—the Australasian Society of Clinical Immunology and Allergy, the Gastroenterological Society of Australia, Australian Society of Pediatric Gastroenterology Hepatology and Nutrition, and the Royal Australian College of General Practitioners—invited their members to assist with the recruitment. The research team also directly contacted specialized EoE clinicians, general practitioners (GPs),

TABLE 1 | Validated tools incorporated in the survey instrument.

Validated tool	Measures
Bakas Caregiving Outcomes Scale© Bakas (2007)* (16)	Life changes in family caregivers. Validated for use in caregivers
PedsQL™ Eosinophilic Esophagitis Module Standard Version 3.0 Parent reports© Varni (2012)* (17)	Parents' perceptions of the HRQoL of their EoE child in the previous month. Validated for use in pediatric EoE for children aged 2–18 years old
PedsQL™ Infant Scales© Varni (1998)* (18)	HRQoL. Validated for use in healthy and ill infants aged 0–24 months
Australian Bureau of Statistics 2016 Census Household Form# (19)	Age, ethnicity, and language spoken at home. Validated for use in the Australian general population
PedsQL™ Family Information Form© Varni (1998)# (20)	Demographic details including the child's date of birth and gender and the impact of EoE on hospital visits, school absences, and parental work absences. Validated for use in pediatric patients with chronic disease
Complementary Medicine Use, Literacy and Disclosure in the Australian Population# (21, 22)	Patterns of CM use; understanding and communication of CM use. Validated for use in the Australian general population
Complementary Therapies Questionnaire# (12)	Experiences and perceptions of CM use including concerns, reasons for use, views on future use, decisions leading to use, and perceived efficacy of CM treatment. Validated for use in pediatric inflammatory bowel disease patients

**Minor amendments were made, including spelling and grammatical changes, to ensure that they were appropriate for an Australian audience and were specific to EoE populations.*
#Specific survey items have also been adopted from other pre-existing validated tools to confirm sociodemographic details (19, 20), patterns of CM use, understanding and communication of CM use patterns (21, 22), and experiences and perceptions of CM use including concerns, reasons for use, views on future use, decisions leading to use, and perceived efficacy of CM treatment (12).
Additional survey items were developed from literature reporting CM and other health service use in chronic inflammatory pediatric diseases, including gastrointestinal disorders (12, 13, 17, 23–37), to confirm eligibility, ensure that that the questions addressed CM use in children not the career, gauge treatment burden [the workload attributed to healthcare and its impact on patient well-being and functioning (38)], and access funding support for EoE patients.

and hospital-based pediatric allergy and gastroenterology departments across Australia and invited their assistance with recruitment. Snowball sampling was used to encourage medical specialists and carers of pediatric EoE children to ask others to participate. The survey incorporated a participant information sheet and a consent statement, with consent implied by survey completion. The participants had the opportunity to win one of 10 AU$50 gift vouchers (*via two* prize draws of five vouchers each) upon survey completion.

Data Collection

Demographic Characteristics

Child age, age at diagnosis, gender, ethnicity, residential postcode, health cover, and carer allowance details were obtained, as well as carer gender and their relationship to the EoE child.

Health Service and Treatment Utilization

The participants were asked to provide information regarding the health services and treatment used by their EoE child, including the recommendation source of each health service and treatment and the frequency of practitioner consultations and associated out-of-pocket expenditure in the previous 12 months. Medicine use, treatments, and practices were separated into pharmaceuticals, CM products, CM therapies, and dietary changes. In accordance with schedule 14 of the Australian Government Therapeutic Goods Regulations 1990, CM products were defined according to their active ingredient, e.g., "a vitamin or provitamin," not by the purpose of usage, i.e., a vitamin deficiency (44).

Data Analysis

Descriptive statistics were determined for each variable. STATA/IC 15 statistical analysis software was used for the data analysis. Missing answers for questions where the respondents were asked to indicate agreement and no other option was provided were classified as "no." All other instances where an answer was not provided were excluded from the analysis. Potential overlap of practitioner type was identified through the participants who provided examples of one practitioner with multiple qualifications, including allergist/immunologist ($n = 3$), dietitian/nutritionist ($n = 1$), and naturopath/nutritionist ($n = 1$). As there was no way to determine if multiple answers were selected for the same practitioner for all respondents, original values were retained. "Other" open-text responses in all categories were reviewed and amendments were made accordingly. For example, where not already allocated to another practitioner, "undergoing hypnotherapy" was allocated to a hypnotherapist, and "once" was reclassified as one to two visits to a practitioner. Where the respondents provided "Other" recommendation sources for child medicines, products, and therapies, that were among the listed options of specified practitioners, the "Other" response was re-classified to the listed category. Where gastroenterologist was listed as "Other," the response was amended to pediatric gastroenterologist. The frequency of visit percentages was calculated from individual "use ever" totals for each health practitioner type. Only respondents who indicated that the use of the medicine, product, and therapy type was for their child's EoE were included in the data analysis. A new variable was also created to represent the total number of practitioner types visited, excluding visits with a "pharmacy or health food store assistant" as this role does not require defined professional or clinical training. Poisson regression analysis was used to determine the relationship between 'time since diagnosis' and use of treatments that have been strongly recommended against (in this study, mast cell stabilizers and antihistamines) (5). Logistic regression analysis was used to determine if private health insurance status was a predictor of CM use for pediatric EoE.

RESULTS

A total of 181 survey responses were included in the analysis after the incomplete survey responses were removed.

Demographics

A total of 232 survey responses were received. Thirty-five incomplete responses were removed, as well as those that did not meet the inclusion criteria ($n = 16$). These included responses with no confirmed EoE diagnosis ($n = 12$), residence outside of Australia ($n = 1$), completion by an EoE child instead of their parent ($n = 2$), and child whose age is over 18 years ($n = 1$). The remaining 181 responses were used for data analysis.

Carer and EoE Child Characteristics

The surveys were almost exclusively completed by a parent ($n = 178$)—in most cases, the mother ($n = 173$) of the EoE child (see **Table 2**). The children were identified predominantly as White/Caucasian (93.3%) and males (71.7%), ages between 13 months and 18 years were represented (mean 9.70; SD 4.67), and the mean time since diagnosis for this study was 4.13 years (SD 3.38; min 0; max 14.17). Most children were covered by a private health insurance (63.3%), but <1/2 of the families received additional financial support from the Australian Government to reduce out-of-pocket medical expenses through a healthcare card (39.4%) or financial support through a carer allowance (27.8%). Private health insurance status was not found to be a significant predictor of overall CM use (practitioner, product, or therapy) for pediatric EoE ($p = 0.86$, OR 1.06; 95% CI: 0.54–2.09), CM practitioner use only ($p = 0.19$, OR 1.54; 95% CI: 0.81–2.94), or CM product or therapy use only ($p = 0.92$, OR 0.97; 95% CI: 0.51–1.85).

Health Service Use

Most children (88.4%) received care from three or more different types of practitioner, with almost two-thirds (60.8%) consulting six or more practitioner types for their EoE. Most children (91.2%) had consulted a medical doctor for their EoE. The most commonly accessed healthcare practitioners at any time for EoE were a pediatric gastroenterologist (86.2%), GP (84.5%), allergist (70.2%), and dietitian (69.6%) (reported in **Table 3**). Almost half of the respondents had consulted a CM practitioner (40.3%) at some time-point after their child's diagnosis, with a naturopath (22.1%) being the most commonly accessed. Most respondents indicated that they saw any type of medical doctor once or twice in the last 12 months. GPs were mostly visited, with more than six visits in the past 12 months (27.0%). Although we are unable to determine if multiple practitioner use was simultaneous or sequential in the previous 12 months, the mean number of different types of practitioner seen for a child's EoE was 4.6 (SD 2.99; min 0; max 12); one-fifth (20.7%) of the respondents indicated that their child had seen both a medical doctor and a CM practitioner.

Use and Perceived Effectiveness of Pharmaceuticals, CMs, and Dietary Changes

Pharmaceuticals (86.2%) were the most commonly used treatment option at any time for pediatric EoE, followed by dietary changes (78.5%), CM products (60.2%), and CM therapies (23.2%) (see **Table 4**). Most respondents indicated that reflux medications (77.9%) had been used for EoE management. Probiotics (43.1%) and nutritional supplements (40.9%) were the most used CM products. Dietary changes were common, with over three quarters (75.1%) of all respondents indicating that they had used elimination diets in the management of their child's EoE, followed by elemental formula (43.7%).

Amongst the pharmaceuticals listed, most respondents (74.4%) perceived corticosteroids as effective, followed by mast cell stabilizers (68.5%). The mast cell stabilizers ($n = 23$) also had the highest percentage (10.5%) of "made worse" responses, over four times greater than each of the other pharmaceuticals. Poisson regression analysis determined that the risk of children using treatments strongly recommended against in EoE therapy, namely, mast cell stabilizers and antihistamines, is 1.6 times greater (CI 1.0–2.6, $p = 0.05$) between 2 and 4 years since diagnosis and 1.9 times greater (CI 1.2–2.9, $p = 0.003$) at 4 years or more after diagnosis when compared with children in their first 2 years since diagnosis. Almost one quarter of the respondents perceived reflux medications ($n = 31$) to be ineffective in EoE management. Despite the small sample sizes for most CM products, the respondents reported high levels of perceived effectiveness for Chinese herbal medicines ($n = 3$, 75.0%) and Western herbal medicines ($n = 5$, 71.4%). Acupuncture was more often perceived to be ineffective ($n = 2$; 33.3%) than effective ($n = 1$; 16.7%). No CM therapy was perceived to have made the child's EoE worse. While most respondents felt that elemental formula was effective (71.2%), 14 respondents found it ineffective and seven were uncertain. The overall effectiveness of the elimination diet (76.2%) was slightly higher than that of the elemental formula.

Sources of Recommendation

All pharmaceuticals used for EoE treatment were predominantly recommended by medical doctors (**Table 5**). Corticosteroids were recommended by a pediatric gastroenterologist in over 80% of cases, as were reflux medications (87.9%). Mast cell stabilizers were only recommended by immunologists, pediatric gastroenterologists, and allergists. All CM therapies were predominantly self-prescribed by the carer (**Table 5**). Medical doctors and other non-CM practitioners were more likely to recommend nutritional supplements and probiotics than any other type of CM. Dietitians recommended nutritional supplements in over 40% of cases, while only 28.4% ($n = 21$) were recommended by pediatric gastroenterologists and 24.3% ($n = 18$) were recommended by CM practitioners. Probiotics were equally recommended by CM practitioners (25.6%) or self-prescribed (25.6%). Elimination diet (69.9%) and elemental formula (62.0%) were mostly recommended by pediatric gastroenterologists, followed by allergists (elimination diet:

TABLE 2 | Sociodemographic characteristics of carers and of EoE children as reported by carers.

Child's characteristics	*n* (%)	Carer characteristics	*n* (%)
Child's gender ($n = 180$)		Carer gender ($n = 180$)	
Male	129 (71.7)	Male	5 (2.8)
Female	51 (28.3)	Female	175 (97.2)
Child's age[#] ($n = 180$)		Carer relationship to child ($n = 179$)	
1–23 months	4 (2.2)	Mother	173 (96.6)
2–4 years	22 (12.2)	Father	5 (2.8)
5–7 years	35 (19.4)	Grandmother	1 (0.6)
8–12 years	69 (38.3)	Carer state of residence ($n = 179$)	
13–18 years	50 (27.8)	Australian Capital Territory	4 (2.2)
Child's age at diagnosis ($n = 181$)		New South Wales	61 (34.1)
1–23 months	45 (24.9)	Northern Territory	1 (0.6)
2–4 years	46 (25.4)	Queensland	46 (25.7)
5–7 years	34 (18.8)	South Australia	14 (7.8)
8–12 years	45 (24.9)	Tasmania	0 (0.0)
13–18 years	11 (6.1)	Victoria	31 (17.3)
Time since diagnosis ($n = 180$)	SD; min–max	Western Australia	22(12.3)
Mean 4.13 years	3.38; 0–14.17	Applied for and approved carer allowance ($n = 180$)	
Child's ethnicity ($n = 180$)	*n* (%)	Yes	50 (27.8)
White/Caucasian	168 (93.3)	No	130 (72.2)
Aboriginal/Torres strait islander	1 (0.6)		
Asian	4 (2.2)		
Middle eastern	2 (1.1)		
Other	5 (2.8)		
Current healthcare card ($n = 180$)			
Yes	71 (39.4)		
No	109 (60.6)		
Current private health insurance ($n = 180$)			
Yes	114 (63.3)		
No	61 (33.9)		
Unsure	5 (2.8)		

[#]*Child's age at survey completion.*

34.6%, elemental formula: 31.7%) and dieticians (elimination diet: 30.2%, elemental formula: 29.1%). Some patients who had used an elimination diet had never seen a dietician or nutritionist (15.4%). Almost one-third of carers self-prescribed (29.4%, $n = 47/160$) non-prescription only pharmaceuticals, CMs, or dietary changes for their EoE child.

DISCUSSION

To our knowledge, this is the first study to explore health service, medicine, and CM use for pediatric EoE. It is difficult to estimate the percentage of the pediatric EoE population in Australia that was captured by this survey as the prevalence rates are changing rapidly (43). The survey was designed in 2017 and undertaken between September 2018 and March 2019. During this time, the prevalence data, based on international (4, 45) and Australian (43) studies, ranged from 1 to 5 in 10,000 and may be as high as 1 in 1,000 in 2020 (2). According to Australian census data (46), it would mean that the survey captured between 4 and 40% of the pediatric EoE population in Australia, depending on what would be considered as accurate prevalence data at the time. A 2018 systematic review and meta-analysis (47), which included 13 studies, focused on HRQoL in patients with EoE of all ages. The sample sizes ranged from $n = 8$ (Australia) (48) to $n = 140$ (USA) (49), emphasizing the large sample size of the study reported here. The EoE children in our study were identified predominantly as White/Caucasian (93.3%) and of male gender (71.7%), which is representative of the general pediatric EoE population (50). The representation by children of all ages (between 13 months to 18 years) and the broad range of time since diagnosis indicate that the responses represented patients at varied stages in their EoE management.

Our study showed that most (86.2%) children had been given a pharmaceutical at some stage to treat their EoE. Reflux medications such as proton pump inhibitors are a first-line treatment option for EoE (7) and were the most commonly used pharmaceutical. However, proton pump inhibitors may be associated with adverse side effects when used for a long term (51), and almost one quarter of those respondents who had used

TABLE 3 | Prevalence and frequency of health service use by EoE children ($n = 181$).

Practitioner type	Use ever *n* (%)[#]	Frequency of visits in the past 12 months *n* (%)				
		None *n* (%)	1–2 *n* (%)	3–4 *n* (%)	5–6 *n* (%)	More than 6 *n* (%)
MEDICAL DOCTORS						
Allergist	127 (70.2)	31 (25.4)	69 (56.5)	14 (11.5)	5 (4.1)	3 (2.5)
General practitioner	153 (84.5)	20 (13.5)	44 (29.7)	31 (21.0)	13 (8.8)	40 (27.0)
Hospital doctor	99 (54.7)	28 (29.8)	30 (31.9)	15 (16.0)	13 (13.8)	8 (8.5)
Immunologist	90 (49.7)	19 (22.9)	47 (56.6)	10 (12.1)	5 (6.0)	2 (2.4)
Pediatric gastroenterologist	156 (86.2)	9 (6.1)	55 (37.2)	45 (30.4)	32 (21.6)	7 (4.7)
Pediatrician	96 (53.0)	34 (37.4)	42 (46.1)	9 (9.9)	4 (4.4)	2 (2.2)
CM PRACTITIONERS						
Acupuncturist	7 (3.9)	5 (71.4)	1 (14.3)	1 (14.3)	0 (0.0)	0 (0.0)
Aromatherapist	3 (1.7)	1 (33.4)	1 (33.3)	1 (33.3)	0 (0.0)	0 (0.0)
Chiropractor	31 (17.1)	13 (46.4)	4 (14.3)	7 (25.0)	0 (0.0)	4 (14.3)
Homeopath	11 (6.1)	5 (50.0)	1 (10.0)	3 (30.0)	1 (10.0)	0 (0.0)
Massage therapist	8 (4.4)	2 (28.6)	2 (28.6)	1 (14.2)	2 (28.6)	0 (0.0)
Naturopath	40 (22.1)	17 (46.0)	11 (29.7)	4 (10.8)	0 (0.0)	5 (13.5)
Osteopath	8 (4.4)	6 (85.7)	0 (0.0)	1 (14.3)	0 (0.0)	0 (0.0)
Relaxation/meditation teacher	7 (3.9)	2 (33.3)	4 (66.7)	0 (0.0)	0 (0.0)	0 (0.0)
Tai chi or qigong teacher	1 (0.6)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Traditional Chinese medicine practitioner	1 (0.6)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Western herbalist	4 (2.2)	3 (75.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (25.0)
Yoga teacher	4 (2.2)	0 (0.0)	2 (66.7)	1 (33.3)	0 (0.0)	0 (0.0)
OTHER HEALTH PRACTITIONERS OR HEALTH WORKERS						
Counselor or other mental health worker	45 (24.9)	10 (23.8)	11 (26.2)	3 (7.1)	7 (16.7)	11 (26.2)
Dietitian	126 (69.6)	44 (36.7)	39 (32.5)	22 (18.3)	9 (7.5)	6 (5.0)
Nutritionist	41 (22.7)	17 (47.2)	12 (33.3)	2 (5.6)	1 (2.8)	4 (11.1)
Pharmacist	74 (40.9)	7 (10.0)	15 (21.4)	11 (15.7)	10 (14.3)	27 (38.6)
Pharmacy or health food store assistant	41 (22.7)	4 (10.5)	11 (28.9)	5 (13.2)	6 (15.8)	12 (31.6)
Other practitioner	20 (11.1)	2 (13.3)	3 (20.0)	2 (13.3)	1 (6.7)	7 (46.7)

[#]*The total number of practitioners is greater than the number of responses as some respondents listed more than one other practitioner.*

them perceived them to be ineffective in EoE management, which is in line with previous findings (52). Proton pump inhibitors can reduce the absorption and bioavailability of nutrients, such as calcium, iron, magnesium, and vitamin B12 (51), which are particularly important in a pediatric population (53). Yet there is limited information on the safety, benefits, and bioavailability of different forms of nutrients, specifically for supplementation in EoE. Mast cell stabilizers and antihistamines, although their use is strongly recommended against for EoE treatment (5), were perceived as effective by most responders. Yet 10.5% of the respondents perceived mast cell stabilizers to have made EoE symptoms worse, over four times greater than reported for each of the other pharmaceuticals. This reflects the need for qualitative interviews to further understand how efficacious treatment is perceived by the parents of children with EoE and for additional research to provide evidence-based treatment options for these patients as well as improved practitioner awareness and education regarding EoE treatment guidelines.

The reported CM use was high, with the respondents indicating that they had consulted with a CM practitioner (40.3%), used CM products (60.2%), or used CM therapies (23.2%) to manage their child's EoE. Nutritional supplements and probiotics were the CM products most commonly recommended by a health professional for EoE, with medical doctors and other non-CM practitioners being more likely to recommend them than any other CM product. As CM products were defined according to their active ingredient (e.g., "a vitamin or provitamin"), not by the purpose of usage (e.g., to correct a deficiency or to supplement in general), supplementation with, e.g., vitamin D, calcium, or iron to correct deficiencies is also counted as CM use. Anecdotally, the wait time for consultations within the Australian public health system [all costs are subsidized by the Australian Government for Australian citizens (39)] for pediatric gastroenterologists and pediatric allergy specialists (allergist/immunologist) can be 12–18 months. Although the waitlists may be reduced for patients opting to consult pediatric gastroenterologists in private practice, out-of-pocket expenses can be higher, particularly for those without a private health insurance cover. Long wait times to access pediatric allergy and gastroenterology specialists

TABLE 4 | Perceived effectiveness of pharmaceuticals, complementary medicines (CMs), and dietary changes for pediatric EoE ($n = 181$).

Medicines, treatments, and practices	Use ever *n* (%)	Perceived effectiveness *n* (%)				
		Very effective *n* (%)	Partially effective *n* (%)	Not effective *n* (%)	Made worse *n* (%)	Unsure *n* (%)
PHARMACEUTICALS						
Antihistamines	107 (59.1)	15 (15.5)	39 (40.2)	24 (24.7)	2 (2.1)	17 (17.5)
Corticosteroids	127 (70.2)	42 (35.9)	45 (38.5)	15 (12.8)	3 (2.6)	12 (10.2)
Mast cell stabilizers	23 (12.7)	8 (42.1)	5 (26.4)	2 (10.5)	2 (10.5)	2 (10.5)
Reflux medications	141 (77.9)	41 (30.8)	45 (33.8)	31 (23.3)	3 (2.3)	13 (9.8)
Other pharmaceuticals^	2 (1.2)					
Any pharmaceuticals^	156 (86.2)					
CM PRODUCTS						
Chinese herbal medicines	4 (2.2)	0 (0.0)	3 (75.0)	0 (0.0)	1 (25.0)	0 (0.0)
Flower essences	14 (7.7)	1 (8.3)	1 (8.3)	5 (41.7)	0 (0.0)	5 (41.7)
Homeopathic medicines	16 (8.8)	0 (0.0)	6 (46.1)	4 (30.8)	2 (15.4)	1 (7.7)
Nutritional supplements	74 (40.9)	6 (9.1)	24 (36.4)	14 (21.2)	2 (3.0)	20 (30.3)
Probiotics	78 (43.1)	5 (7.3)	21 (30.4)	17 (24.6)	6 (8.7)	20 (29.0)
Western herbal medicines	7 (3.9)	2 (28.6)	3 (42.8)	2 (28.6)	0 (0.0)	0 (0.0)
Other complementary medicines^	3 (1.7)					
Any complementary medicines^	109 (60.2)					
CM THERAPIES						
Acupuncture	6 (3.3)	0 (0.0)	1 (16.7)	2 (33.3)	0 (0.0)	3 (50.0)
Aromatherapy	18 (9.9)	1 (6.7)	6 (40.0)	3 (20.0)	0 (0.0)	5 (33.3)
Massage	12 (6.6)	0 (0.0)	4 (40.0)	4 (40.0)	0 (0.0)	2 (20.0)
Relaxation techniques/meditation	25 (13.8)	1 (4.8)	13 (61.9)	7 (33.3)	0 (0.0)	0 (0.0)
Yoga	5 (2.8)	0 (0.0)	3 (75.0)	1 (25.0)	0 (0.0)	0 (0.0)
Other complementary treatments^	6 (3.3)					
Any complementary treatment^	42 (23.2)					
DIETARY CHANGES						
Elemental formula	79 (43.7)	34 (46.6)	18 (24.6)	14 (19.2)	0 (0.0)	7 (9.6)
Elimination diet	136 (75.1)	46 (36.5)	50 (39.7)	14 (11.1)	11 (8.7)	5 (4.0)
Other dietary changes^	2 (1.1)					
Any dietary changes^	142 (78.5)					

^*Perceived effectiveness not available as in some cases one option was chosen for multiple "other" medicines, treatments, and practices.*

within the Australian public health system but easy access to a CM practitioner and products may enhance CM use in this population. Given that carers of children with chronic inflammatory gastrointestinal disease expect the practitioners to be knowledgeable about CM use (54), further research into commonly used CMs for pediatric EoE and education are required so that all practitioners involved in the care are enabled to give evidence-based advice.

The participants in our study reported perceiving some pharmaceuticals and CM products to lack efficacy or worsen symptoms. While there may be several reasons for these results, including worsening of symptoms due to the use of an ineffective treatment, they warrant further investigation in consumer interviews. Inadvertent exposure to an antigenic EoE or IgE allergy trigger can occur due to the inadequate health literacy of the carer, poorly executed elimination diet, or undisclosed excipient ingredients in the medicine itself. For example, otherwise effective medicines may be perceived as ineffective due to containing unknown excipients such as milk proteins, soy, wheat, corn, rice, and potato, which can be common EoE antigens (55). Depending on therapeutic regulations, this information may be omitted from product labeling (56, 57). It is therefore vital to raise awareness and knowledge among clinicians and self-prescribing carers of EoE children about medicine excipients and engage the expertise of pharmacists or other stakeholders to reduce the risk of exposure to known EoE triggers.

Our study also found that almost one-third of carers self-prescribe non-prescription-only pharmaceuticals, CMs or dietary changes for their EoE child. The importance of carers in the management of pediatric EoE and the selection of treatment options should not be underestimated. Consequently, practitioners should facilitate open discussions with carers regarding their complete medicine and treatment use for their EoE child. With limited research into the efficacy and safety of EoE treatment and management options, parent perceptions, experiences, and decisions provide valuable insights (58), which

TABLE 5 | Source of recommendation for pharmaceuticals, complementary medicines (CMs), and dietary changes for pediatric EoE ($n = 181$).

Pharmaceuticals, CMs, and dietary changes	Source of recommendation *n* (%)*												
	Allergist	Immunologist	Pediatric gastro-enterologist	Pediatrician	General practitioner	Hospital doctor	Pharmacist	Dietitian or nutritionist	Counselor or other mental healthcare worker	CM^ practitioner	Pharmacy or health food store assistant	Family or friend	Self-prescribed
	n (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)
PHARMACEUTICALS													
Antihistamines ($n = 107$)	54 (50.5)	29 (27.1)	24 (22.4)	12 (11.2)	32 (29.9)	8 (7.5)	4 (3.7)	2 (1.9)	0 (0.0)	1 (0.9)	0 (0.0)	3 (2.8)	4 (3.7)
Corticosteroids ($n = 127$)	22 (17.3)	13 (10.2)	102 (80.3)	7 (5.5)	7 (5.5)	6 (4.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Mast cell stabilizers ($n = 23$)	6 (26.1)	10 (43.5)	9 (39.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Reflux medications ($n = 141$)	21 (14.9)	11 (7.8)	124 (87.9)	21 (14.9)	22 (15.6)	3 (2.1)	0 (0.0)	1 (8.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
CM PRODUCTS													
Chinese herbal medicines ($n = 4$)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (75.0)	0 (0.0)	1 (25.0)	0 (0.0)
Flower essences ($n = 14$)	1 (7.1)	0 (0.0)	0 (0.0)	0 (0.0)	1 (7.1)	0 (0.0)	1 (7.1)	1 (7.1)	0 (0.0)	5 (35.7)	0 (0.0)	1 (7.1)	8 (57.1)
Homeopathic medicines ($n = 16$)	0 (0.0)	1 (6.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (6.3)	0 (0.0)	0 (0.0)	9(56.3)	0 (0.0)	2 (12.5)	2 (12.5)
Nutritional supplements ($n = 74$)	10 (13.5)	6 (8.1)	21 (28.4)	4 (5.4)	10 (13.5)	1 (1.4)	2 (2.7)	33 (4.6)	0 (0.0)	18 (24.3)	1 (1.4)	1 (1.4)	10 (13.5)
Probiotics ($n = 78$)	7 (9.0)	6 (7.7)	11 (14.1)	3 (3.9)	15 (19.2)	0 (0.0)	4 (5.1)	17 (21.8)	0 (0.0)	20 (25.6)	2 (2.6)	2 (2.6)	20 (25.6)
Western herbal medicines ($n = 7$)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (14.3)	0 (0.0)	0 (0.0)	5 (71.4)	0 (0.0)	2 (28.6)	1 (14.3)
CM THERAPIES													
Acupuncture ($n = 6$)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (33.3)	0 (0.0)	0 (0.0)	4 (66.7)
Aromatherapy ($n = 18$)	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	7 (38.9)	1 (5.6)	3 (16.7)	6 (33.3)
Massage ($n = 12$)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)	1 (8.3)	0 (0.0)	1 (8.3)	0 (0.0)	1 (8.3)	3 (25.0)	0 (0.0)	0 (0.0)	3 (25.0)
Relaxation techniques/Meditation ($n = 25$)	1 (4.0)	0 (0.0)	1 (4.0)	1 (4.0)	2 (8.0)	1 (4.0)	0 (0.0)	0 (0.0)	7 (28.0)	2 (8.0)	0 (0.0)	0 (0.0)	11 (44.0)
Yoga ($n = 5$)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (20.0)	1 (20.0)	0 (0.0)	0 (0.0)	1 (20.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (40.0)
DIETARY CHANGES													
Elemental formula ($n = 79$)	25 (31.7)	9 (11.4)	49 (62.0)	9 (11.4)	4 (5.1)	3 (3.8)	0 (0.0)	23 (29.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.3)
Elimination diet ($n = 136$)	47 (34.6)	21 (15.4)	95 (69.9)	10 (7.4)	3 (2.2)	3 (2.2)	0 (0.0)	42 (30.9)	0 (0.0)	7 (5.2)	0 (0.0)	0 (0.0)	6 (4.4)

**Total sources of recommendation may be different than the number of users if the respondents selected more than one or no recommendation source.*

^Includes acupuncturist, aromatherapist, chiropractor, homeopath, massage therapist, naturopath, osteopath, relaxation/meditation teacher, tai chi or qigong teacher, traditional Chinese medicine practitioner, Western herbalist, and yoga teacher.

are worthy of increased attention. Parental proxy report in young children with EoE can function as an adequate marker for child self-reported symptoms and HRQoL measures (59). Additionally, parent involvement in decision making has been shown to improve a child's treatment outcomes (60), suggesting that carers play an important role in disease management and should be seen as treatment partners by the practitioners. However, due to the scarcity of evidence-based treatment options in EoE, it is challenging for the practitioners to effectively fulfill the carers' and the patients' expectations and needs.

Elimination diets were commonly used by study participants and were mostly reported as being recommended by pediatric gastroenterologists. Decisions surrounding the choice and the implementation of dietary elimination and re-introduction are complex and can result in treatment failure or symptom worsening, potentially due to factors such as inadequate patient education, non-adherence, and atypical individual triggers (61, 62). Unfortunately, at least one in seven respondents who had used an elimination diet had never consulted a dietitian or nutritionist. The reason may lay in the fact that neither profession is classified as a registered health profession in Australia (63), leading to a lack of clarity surrounding the education, qualification, and professional standards of these professions for consumers. As EoE is one of many diseases requiring expert dietary management, it is imperative that these professions are regulated through professional registration which would result in the implementation of mandatory educational and practice standards, leading to enhanced trust and acceptance by consumers and therefore most likely to higher consultation rates. There is evidence that gastroenterologists often agree to patient-driven elimination diets without dietitian support and do not adhere to the recommendations for repeated biopsies to monitor ongoing response to therapy (64). This underutilization of dietitians and nutritionists in our study may reflect a lack of referral by gastroenterologists, allergists, and immunologists or a scarcity of practitioners with specialized knowledge; hence, identifying and accessing them may prove challenging for both the referring practitioners and the carers. Given that elimination diets are first-line treatment options in EoE and the high percentage of EoE patients using them, it is paramount to increase workforce education and educational resources and encourage collaboration between all practitioners to establish a wider referral network and provide specified support for EoE patients.

The necessity of collaboration between practitioners and the close communication with carers is particularly warranted as most children received care from three or more different practitioner types, with almost two-thirds having seen six or more different types of practitioner for their EoE. This high rate of diversity in practitioner types accessed for children with EoE is congruent with existing data on healthcare utilization by children with a rare disease (65). The parents of children with a rare disease often feel isolated and under-supported and perceive that there is poor coordination between care providers, requiring the parent to fill multiple roles and become the "expert" in the care of their child (65, 66). This social burden may be amplified by the financial burden to carers. In Australia, private health insurance can cover specific cost related to private hospital treatment and other medical services, which could include certain CMs (40). Our study shows that a higher percentage of EoE patients (63.3%) have private health insurance than is seen in the general Australian population (53.5%) (67). Carer allowance is available for those persons who provide additional daily care to a child who has a serious chronic illness (41). Healthcare cards can reduce the cost of certain prescription medications and medical doctor consultations and are automatically issued to those persons receiving various government payments or subsidies, including carer allowance (42). Our study showed that nearly two-thirds of the children did not have a healthcare card and a third of the respondents did not have private health insurance to reduce out-of-pocket expenses. Further exploration of financial burden is needed as existing data indicate that EoE-related costs are striking and consistently higher than those of healthy consumers (10, 47). The economic impact of poorly coordinated care encompassing the possible duplication of services as parents attempt to meet the healthcare needs of their family must be carefully considered. Collaboration between healthcare practitioners is thus even more important as it can help to identify the areas of unnecessary expenditure for patients and reduce financial barriers to treatment.

There are several study limitations. Only respondents involving a child diagnosed with EoE who had undergone an endoscopy were included in the analysis. An endoscopy is predominantly performed by a pediatric gastroenterologist; however, not all respondents indicated that their child had seen a pediatric gastroenterologist (or other medical doctor) for their EoE. This may be reflective of some respondents including only consultations post-EoE diagnosis and results in perceived lower numbers of pediatric gastroenterologist and other practitioner consultations as they occurred prior to or during the diagnosis process. Additionally, as the question regarding practitioner use ever required respondents to indicate agreement and no other option was provided, missing answers were classified as "no." Therefore, practitioner visits may be under-reported due to missing answers. This study was based on self-reports and may therefore be subject to recall bias. Questions surrounding the efficacy of medications and therapies are perceptions of the carer only and may not be a true reflection of histological change in EoE.

CONCLUSIONS

This study identified a large variety of practitioners who are involved in the care of EoE patients, resulting in a diverse range of treatment options being recommended and accessed and in a possible treatment-related burden. In addition, carer involvement in the choice of treatment for pediatric EoE is high. Referrals and collaboration between healthcare providers as well as education and shared decision making with carers are required to successfully navigate this complex disease and provide adequate care for children with EoE. The high rate of CM use, particularly given the absence of EoE guidelines in

Australia, warrants further attention by clinicians, policy makers, and researchers.

DATA AVAILABILITY STATEMENT

The research data are stored securely as per Griffith University ethics approval and cannot be made publicly available. The authors will consider any reasonable request for access to the anonymized data according to the privacy statement provided with information and consent materials. Please direct any requests to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Griffith University Human Research Ethics Committee (#2018/120). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

NH drafted the manuscript. All authors contributed to the study design, data analysis, and interpretation, provided editorial comments, read, and approved the final manuscript.

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