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Infant formula supplemented with polyamines alters the intestinal microbiota in neonatal BALB/cOlaHsd mice☆,☆☆,★

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Abstract

Polyamines play a critical role in the development of intestinal and immune systems during the infant breastfeeding period, but the effect of polyamines on the microbiota has not been reported. The aim of our study was to characterize the impact on the colonization pattern in neonatal BALB/cOlaHsd mice after supplementing an infant formula (IF) with a mixture of putrescine (PUT), spermidine (SPD) and spermine (SPM). A total of 48 pups (14 days old) were randomly assigned to 4-day intervention groups as follows: breast-fed (unweaned) pups (n=12); weaned pups (n=12) fed an infant formula (IF); weaned pups (n=12) fed an IF enriched with a low concentration of PUT, SPD and SPM (2.10, 22.05 and 38.00 μg/day, respectively); and weaned pups (n=12) fed with IF enriched with a high concentration of PUT, SPD and SPM (8.40, 88.20 and 152.00 μg/day, respectively) of polyamines in accordance with normal proportions found in human milk. Microbiota composition was analyzed by fluorescent in situ hybridization (FISH) with flow cytometry detection. Microbiota changes in formula-fed mice were significantly greater following supplementation with polyamines (P<.01). *Bifidobacterium* group bacteria, *Akkermansia*-like bacteria and *Lactobacillus–Enterococcus* group levels were higher in the groups fed infant formula supplemented with polyamines, resulting in even higher numbers of bacteria than in the breastfed pups. Our findings indicate that infant formulas enriched with polyamines may interact with gut microbiota, suggesting that further studies in human infants are required to assess the impact of polyamines on both growth and microbiota levels.

Keywords: Polyamines; Putrescine; Spermidine; Spermine; Microbiota; BALB/c; Infant formula; Breastfeeding

1. Introduction

In all mammals, maternal milk is the key source of nutrition during the early developmental phase. Human milk is a complex composition of nutrients and bioactive components, such as nucleotides, hormones, growth factors and anti-inflammatory and immunomodulatory agents [1,2], designed to fulfil the needs of the growing young infant. Protective compounds, such as cytokines, oligosaccharides and even microbes, in breast milk provide the newborn with the means to adapt to the environment [3,4]. Among other compounds detected in human milk, polyamines, such as spermidine, spermine and putrescine, are of great interest due to their reported biological roles in eukaryotic cells, stimulating cellular proliferation and differentiation [5]. Moreover, there is evidence that polyamines participate in several processes related to the immune system, including immune system development [6,7], inflammatory response modulation [8,9] and normal function of the immune system [10]. Polyamines may also have a potential role in the growth and development of the digestive tract wall and colonic mucosa in neonatal mammals [11]. Other reports suggest that they participate in the maintenance of intestinal mucosal integrity by regulating epithelial barrier functions through expression of E-cadherin [12,13]. High levels of spermine and spermidine have been shown to be immunoprotective by decreasing the permeability of the intestinal mucosa [14]. These factors suggest that such components may have an important role in the development of gut microbiota and the immune system.

There is increasing evidence that breastfeeding has both short- and long-term beneficial effects on the infant and provides an optimal

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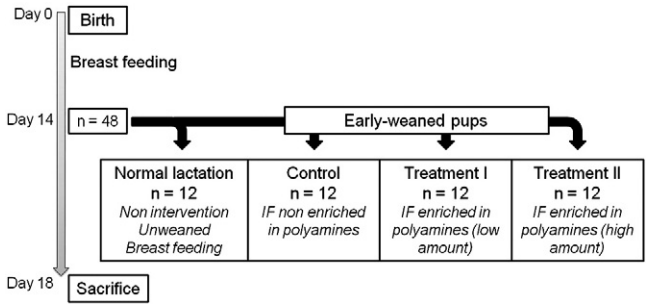

Fig. 1. Experimental study design.

source of nutrition. The most important effects are improved cognitive development, reduced incidence of immune-related diseases (e.g., allergies, type 1 diabetes and inflammatory bowel disease) and protection from infections [15]. Furthermore, polyamines have also demonstrated that they affect the allergy response, thereby increasing the protective effect of breast milk [14,16]. Due to the benefits it provides, exclusive breastfeeding during the first months is recommended by the World Health Organization [17]. When breastfeeding is not possible, infant formula may be the only option for mothers.

The mean polyamine concentrations of 0.021, 0.320 and 0.633 ppm have been reported in human milk for PUT, SPD and SPM, respectively. The concentrations may vary depending on factors such as ethnic origin, diet and age of the mother or lactation stage [18-20]. However, the content of polyamines in current infant formulas (IF) is low, about 10 times less than in breast milk [18]. This may be partly due to the high polyamine oxidase activity and diamine oxidase activity in IF [5], which resist thermic treatments during processing. Thus, supplementation with polyamines may ensure better nutrition of infants and ensure the inclusion of bioactive factors present in breast milk making IF more similar to human milk.

We hypothesised that polyamines, considered as one bioactive factor that acts on infant health impacting gastrointestinal tract development and maturation and the immune system, could modulate microbial colonization patterns. To test this hypothesis, the aim of this study was to assess the impact of supplementation of an IF with a mixture of different polyamines (PUT, SPD and SPM) in concentrations present in breast milk on the neonatal microbiota compositions in BALB/cOlaHsd mice.

2. Material and methods

2.1. Animals

A total of 48 pups, derived from a breeding colony of BALB/cOlaHsd mice supplied by Harlan Laboratories (Horst, Netherlands), were used in this study. The progenitor mice were 8 weeks of age and were acclimatized for 30 days prior to breeding. All the mice were determined to be healthy on the basis of individual physical examinations and pathogen free based on results of the routine microbiological screening performed in the colony in accordance with European recommendations [21].

All the mice were maintained in stainless steel Eurostandard Type II cages (36.5×20.7×14.0 cm) protected with filter tops. The cages had solid bottoms, were covered with Aspen chip bedding (Tapvei, Kaavi, Finland) and were provided with some nesting material. Cage changes for the adult mice were undertaken twice a week, but never during the study period using the pups. The environment in the room consisted of a temperature range of 23°C (±3°C), a relative humidity of 55 ±15% and an artificial illumination of a 12-h light/dark cycle (lights on at 0600). They were maintained on a standard laboratory diet [RM3 (E) SOYA-FREE, product code 801710, Special Diets Services, Witham, Essex, UK] and were allowed free access to water.

Day of birth was referred to as Day 0 of neonatal life. At the commencement of the study, all the pups were 14 days of age, and their mean body weight was 7.94±1.01 g. Throughout the study period, all the pups in the breastfed group had free access to the dam's nipples, and all the pups in the other groups had free access to a standard moist diet consisting of a porridge made by adding warm water to an infant formula at a final proportion of 65 g of infant formula/15 ml of water.

All the adult mice were fed a standard mouse chow [RM3 (E) SOYA-FREE] *ad libitum*. Tap water was provided without restrictions to both adult and infant mice in polycarbonate bottles.

The animals were weighed daily during the intervention study, and handling was done in the same time range to avoid the influence of biological rhythms.

This study was performed at the Central Animal Laboratory, University of Turku, Finland. Pilot experiments were performed to optimize all the experimental procedures including handling and treatments.

The experimental protocol was approved by the National Ethics Committee for Animal Experiments in Finland. The mice were handled in accordance with Finnish legislation and the Council of European Convention ETS 123 on the use of vertebrate animals for scientific purposes.

2.2. Study design

The animals were randomly assigned to dietary intervention groups and treated as described in Fig. 1. Intervention was given for 4 days. The study groups were breastfed (unweaned) pups (normal lactation, $n=12$); weaned pups fed on IF (control, $n=12$); and weaned pups fed on IF enriched with low (Treatment I, $n=12$) and high (Treatment II, $n=12$) concentrations of polyamines.

Table 1
Nutritional composition of infant formula

Energetic value	kJ	2158	Vitamin A	μg	523.0
	kcal	516	Vitamin D	μg	7.8
Proteins	g	10.2	Vitamin E	mg	7.8
Casein	g	5.1	Vitamin K	μg	52.0
Serum	g	5.1	Vitamin C	mg	52.0
Carbohydrates	g	56.9	Vitamin B_1	μg	523.0
Fats	g	27.5	Vitamin B_2	μg	785.0
Linoleic acid	mg	4129.5	Niacin	mg	5.2
Linolenic acid	mg	311.8	Vitamin B_6	μg	523.0
Na	mg	154.0	Folic acid	μg	78.0
K	mg	495.0	Vitamin B_{12}	μg	2.1
Cl	mg	326.0	Biotin	μg	16.0
Ca	mg	387.0	Pantothenic acid	μg	2353.0
P	mg	246.0	L-Carnitine	mg	7.8
Ca/P balance		1.6	Taurine	mg	42.0
Mg	mg	46.0	Inositol	mg	26.1
Fe	mg	6.6	Choline	mg	63,0
Zn	mg	4.2	Adenosine 5′-monophosphate	mg	3,6
Cu	μg	314.0	Cytosine 5′-monophosphate	mg	12,4
I	μg	78.0	Guanosine 5′-monophosphate	mg	2,1
Se	μg	7.0	Uridine 5′-monophosphate	mg	6,5

Values are expressed as per 100 g of commercial dry product.

Table 2
Sequences of the probes used in FISH

Probe	Target	Sequence from 5′ to 3′	Hybridisation temperature (°C)	Reference
EUB 338	Total bacteria	GCT GCC TCC CGT AGG AGT	50	Amann et al., 1990 [28]
Bif 164	*Bifidobacterium*	CAT CCG GCA TTA CCA CCC	50	Langendijk et al., 1995 [29]
Bac 303	*Bacteroides–Prevotella*	CCA ATG TGG GGG ACC TT	45	Manz et al.. 1996 [30]
Chis 150	*Clostridia* subgrp. *perfringens/Histolyticum*	TTA TGC GGT ATT AAT CTY CCT TT	50	Franks et al., 1998 [31]
Lab 158	*Lactobacillus–Enterococcus*	GGT ATT AGC AYC TGT TTC CA	45	Harmsen et al., 1999 [32]
Muc 1437	*Akkermansia*-like bacteria	CCT TGC GGT TGG CTT CAG AT	50	Collado et al., 2007 [22]

Y represents a (C/T) wobble nucleotide.

The unweaned group was caged during the study in pairs (one male and one female) with a mother. In the weaned groups, the pups were caged in pairs (one male and one female) with a 28–32-day-old female mouse who acted as trainer to teach them how to eat and drink. Infant formula, both nonenriched and enriched with polyamines, was administered to the control and treatment groups, respectively, by oral gavage twice daily. Handling was done in the same time range to avoid the influence of biological rhythms.

After the 4-day diet intervention, the animals were anesthetized with isoflurane and samples were collected. Immediately after euthanasia by cervical dislocation, the entire intestinal tract was removed from the pups on Day 18. The full contents of the large intestines were emptied and diluted [1:10 (w/v)] in sterile phosphate buffered saline (PBS). Then, one volume was transferred into three volumes of 4% paraformaldehyde and fixed at 4°C overnight. After fixation, the intestinal contents were centrifuged (12,000×*g*, 3 min, 4°C) and washed with PBS three times [22]. Then, bacterial pellets were stored in PBS–ethanol (1:1) at −20°C until analyzed.

2.3. Formula and formula supplemented with polyamines

Infant formula was supplied by Hero España (Alcantarilla, Spain). The basic formula was designed according to the European Infant Formula directive and the ESPGHAN recommendations [23,24]. It was a commercial IF targeted for babies during the first 6 months, fortified with nucleotides, α-lactalbumin, and ω-3 and ω-6 fatty acids (Table 1).

Nonenriched formula and formula with polyamines (100 μl) were made with warm water following the manufacturer's instructions and given to the pups twice daily by oral gavage. The polyamines tested in the study were putrescine (D13208, Aldrich, Steinheim, Germany), spermidine (S2626, Sigma, Steinheim, Germany) and spermine (85590, Fluka, Steinheim, Germany). The concentration levels tested were 2.10 μg/day PUT, 22.05 μg/day SPD and 38.00 μg/day SPM for the low concentration group, and for the high concentration group the levels were 8.40 μg/day PUT, 88.20 μg/day SPD and 152.00 μg/day SPM — Treatment II was four times higher than Treatment I. The polyamines were prepared in water solution and kept refrigerated at 4°C until addition to the IF. The amount of polyamines for each group was added to the IF immediately before administering it to the mice to avoid degradation by polyamine oxidase. The proportion between the different polyamines (3.38% for PUT, 35.48% for SPD and 61.14% for SPM) was based on the proportion of these polyamines found in human milk [18-20], and daily intake was lower than nonobserved adverse effect levels [25].

Table 3
Bacterial counts as percentage hybridised with each group-specific probe relative to total bacteria hybridised with the EUB 338 probe

	Treatment			
	Normal lactation	Control	Treatment I	Treatment II
Bacteroides/Prevotella	57 ± 14^{a}	20 ± 8^{c}	35 ± 8^{b}	49 ± 12^{a}
Bifidobacterium	21 ± 5^{b}	10 ± 7^{c}	27 ± 8^{ab}	34 ± 13^{ab}
Clostridia subgrp. *perfringens/Histolyticum*	11 ± 5^{ab}	5 ± 3^{c}	12 ± 4^{ab}	17 ± 11^{ab}
Lactobacillus/Enterococcus	9 ± 3^{bc}	6 ± 3^{c}	13 ± 3^{ab}	16 ± 4^{a}
Akkermansia-like bacteria	15 ± 2^{b}	11 ± 2^{c}	20 ± 6^{ab}	27 ± 8^{a}

Data are expressed as mean±S.D. (n=12). Significant differences among microbial counts are shown with letters; groups with different letters (a, b, c) in the same row have statistically significant differences in bacterial group population at a level of P<.01.

2.4. Microbiota composition

The culture-independent analysis of the microbiota was carried out by fluorescent in situ hybridization (FISH) combined with flow cytometry (FCM-FISH) using 16S rRNA oligonucleotide probes on 96-well plates. FCM-FISH was performed as described previously [22,26]. Determination of specific bacteria was performed by combining each of the group-specific Cy3 probes with the EUB 338-FITC probe and counting double-positive cells [27]. Specific bacterial group probe sequences are presented in Table 2. All oligonucleotides were purchased from Thermo Electron Corporation (Bioscience Technologies Division, Ulm, Germany).

Data acquisition was performed with an LSR II flow cytometer equipped with an HTS 96-well plate reader (Becton Dickinson, San Jose, CA, USA). A 15-mV argon ion laser (488 nm) was used to measure forward and side scatter (488±10 nm), green fluorescent for FITC (530±30 nm) and red fluorescent for Cy3 (575 ±26 nm). Forty microliters of the samples was collected in duplicate for each sample. Data were analysed with BD FACSDiva software (Becton Dickinson). Results were expressed as cells hybridising with the group-specific Cy3 probes as a proportion of the total bacteria hybridising with the EUB 338-FITC bacteria domain probe.

2.5. Statistical analysis

Statistical analysis was performed using SPSS software version 15.0. The analysis for normal distribution of the data was performed by a Kolmogorov–Smirnov test. One-way ANOVA and Tukey's and Games–Howell post hoc tests were used. Significant differences were considered at $P\leq.05$.

3. Results

The proportions of cells hybridised with the group-specific probes among the bacteria detected with the EUB 338 probe are presented in Table 3. The box-and-whisker diagrams of the bacterial group populations are presented in Figs. 2 and 3.

The proportions of the bacterial groups characterised were increased by polyamine enrichment of the IF to similar or higher levels as those found in the breastfed group and to higher levels than in the control group.

Bifidobacterium, *Bacteroides-Prevotella* and *Clostridium* groups were the most predominant groups in the large intestines of the mice. They were significantly higher in the treatment and normal lactation groups than in the formula-fed pups (P<.01), as shown in Figs. 2 and 3.

Lactobacillus–Enterococcus group levels were also higher in the breastfed group than in the control group (P<.001). The microbial levels detected in the formula-fed group increased with the supplementation of polyamines, and the highest cell counts were detected in the high polyamine concentration group (Fig. 3). However, *Bifidobacterium* group and *Lactobacillus* group cell counts were higher in the infant formula supplemented with polyamine groups, differing from the levels observed in the breastfed pups.

Akkermansia-like bacteria populations in the different groups displayed the same behaviour as *Bifidobacterium*, which could be related to the healthy status of the intestinal tracts.

Microbial levels between the breastfed group and the infant formula supplemented with higher concentration of polyamines were

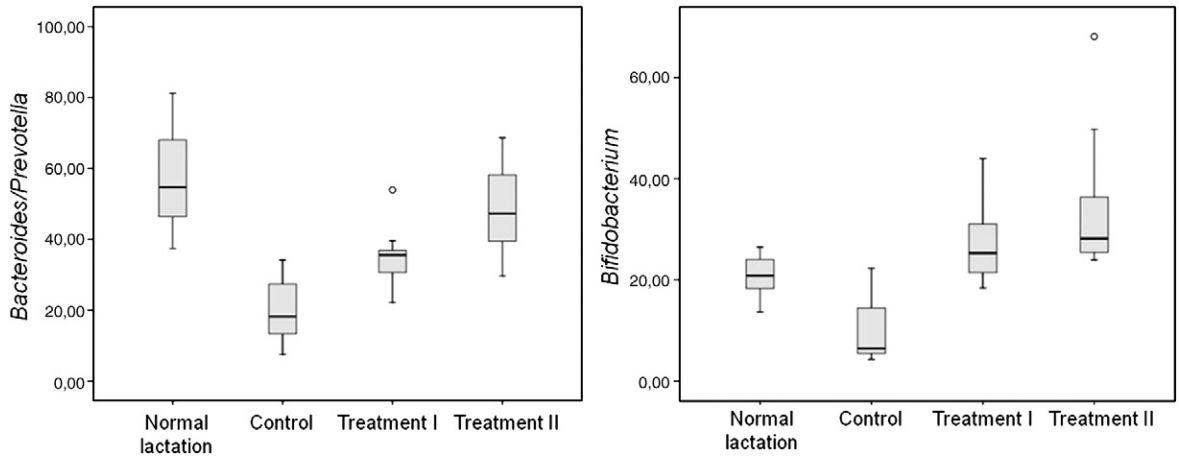

Fig. 2. Box-and-whisker diagram of *Bacteroides/Prevotella* and *Bifidobacterium* percentage levels found in the contents of the large intestines. Each bar represents the smallest observation, lower quartile (Q1), median, upper quartile (Q3) and largest observation. The circles represent outlier data.

similar, while differences with the formula-fed group were detected (Table 3 and Fig. 3).

Increasing amounts of polyamine supplementation appeared to increase the relative population of all microorganisms studied. Animals fed with Treatment II had higher bacterial levels than animals fed with Treatment I, but they were only statistically significant for the *Bacteroides–Prevotella* group (Table 3).

No sex differences were found.

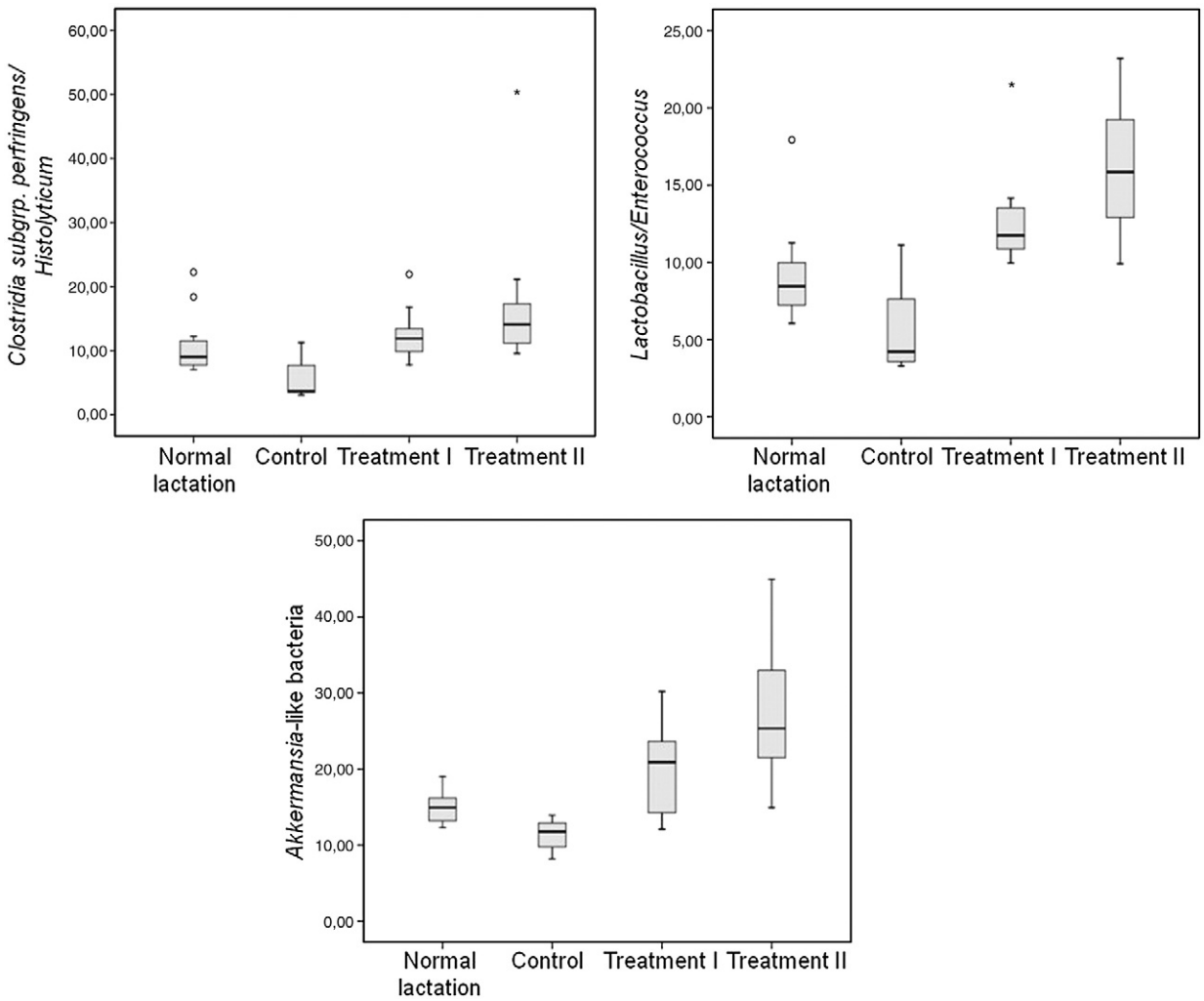

Fig. 3. Box-and-whisker diagram of *Clostridia* subgrp. *Perfringens/Histolyticum, Lactobacillus/Enterococcus* and *Akkermansia*-like bacteria shows the percentage levels found in the large intestines. Each bar represents the smallest observation, lower quartile (Q1), median, upper quartile (Q3) and largest observation. The circles and stars represent outlier data.

4. Discussion

To our knowledge, this is the first study demonstrating that supplementation of infant formula with polyamines has a significant impact on the gut microbiota composition and activity in neonatal BALB/cOlaHsd mice. The results demonstrated that polyamines in infant formula interact with microbiota development, and microbiota composition in the supplemented formula groups resembled closely that of the breastfed groups.

Although intake of dietary polyamines has been known to have a direct impact on health, limited data are available on polyamine content in foods and their effects. Polyamines are common components present in human breast milk, the preferred and recommended source of nutrition for infants. With the early core microbiota formation being dependent on exposure to the microbes that first colonise the gastrointestinal tract, the establishment of a 'healthy' microbiota in early life is likely to be critical for normal development. In the present study, we assessed the effect of infant formula supplementation with polyamines on the intestinal microbiota composition. In this study, the most predominant groups found in mouse large intestinal contents are similar to those found in human infants [33,34]. These bacteria belong to groups which may be associated with mucin, and mucin production could be correlated with an increase of these microorganisms.

The high *Bifidobacterium* levels found in the large intestines of the pups fed with IF enriched with polyamines could be a biological index of the health status of the intestinal tract [35]. These results suggest that polyamines increase the number of *Bifidobacterium* species in the intestine and promote a healthy mucosal status. Bifidobacteria are the predominant microbiota of healthy breast-fed infants and are considered to be a hallmark of a healthy breastfed infant. Bifidobacteria have a biological role in mucosal host-microbe crosstalk, immune regulation and inflammation control [15,36]. Compared with formula, human milk is of a more complex composition, providing both optimal nutrition for the newborn and protective nutrients that contribute to the development of mucosal defences.

A recent study reported that high numbers of bifidobacteria may correlate positively with the normalization of inflammatory status and improved glucose tolerance and glucose-induced insulin secretion [37]. Therefore, infant formula supplemented with polyamines might ensure improved nutrition for infants, mimicking the effects of breastfeeding and ensuring the inclusion of bioactive factors present in breast milk. The enrichment of a normal infant formula with polyamines in the same proportion as human milk may increase the relative populations of bifidobacteria in the large intestine to a comparable level as healthy breastfed infants. There are three possible explanations for this: (1) polyamines, which are reported to be cellular growth modulators [10,38], increase specifically the proliferation of these bacterial groups; (2) polyamines have an inhibitory effect against other bacterial groups, made possible by a higher proliferation of this bacterial group; and/or (3) the stimulation of the immune system exerted by polyamines allows a greater spread of these beneficial host microbes. Thus, strategies targeting gut microbiota modulation in favour of the bifidobacteria group could be a useful tool for improving health in early as well as in later life. Moreover, *Bifidobacterium* species have been suggested as the key biological markers of a healthy breastfed infant gut; thus, it is important to analyze further bifidobacteria species composition. Differences in *Bifidobacterium* species composition have been related to inflammatory diseases such as allergy, metabolic disorders and obesity [39,40], and different immunomodulatory properties have been attributed to different *Bifidobacterium* species.

We found lower levels of the *Lactobacillus–Enterococcus* group in formula-fed neonatal BALB/cOlaHsd pups than in those that were breastfed or were fed polyamine-supplemented formulas. Together with bifidobacteria, the *Lactobacillus* group is one of the target groups in the intestinal microbiota of breast-fed infants, whereas infants who receive cow's milk-based infant formulas, which naturally contain low levels of oligosaccharides, often have higher concentrations of potentially pathogenic bacteria, such as *Enterobacteriaceae* and *Clostridia*, in their intestinal microbiota [41]. However, scarce information about the dietary impact on *Lactobacillus* group levels is available.

Our results showed that pups fed IF enriched with polyamines and breastfed pups had higher levels of *Akkermansia*-like bacteria in their large intestines. *Akkermansia muciniphila* has been shown to be a common member of the gut microbiota of human and animals and has the capability to utilise intestinal mucus as a source of nutrients at the same time promoting the development of innate and adaptive immune responses [41]. The high prevalence of *Akkermansia*-type bacteria in diverse gut ecosystems also supports its nonpathogenic nature, and its presence is probably important for equilibrium of the microbial ecosystem. *A. muciniphila* has so far not been correlated with any disease or sign of pathogenicity [42]. A recent study [43] reported that it was negatively correlated with acute appendicitis, together with *Faecalibacterium prausnitzii, Eubacterium rectale* and *Bacteroides* spp, inhabitants of the intestinal ecosystem. Although several studies have reported the potential involvement of mucin-degrading bacteria, such as *A. muciniphila*, in the pathogenesis of inflammatory diseases [44,45] and in animal models, higher levels of *Akkermansia* have also been associated with allergic diarrhoea, while lower levels were associated with diets with lower allergic symptoms and fasting in mice [46,47]. Further studies are necessary to clarify the roles of and variations in *Akkermansia*-like bacteria populations in health-disease status.

Our results demonstrated that polyamine supplementation of infant formula had an effect on all bacterial groups analyzed in similar amounts to what we reported for normal lactation. Differences between Treatment Groups I and II including an increase in *Bacteroides–Prevotella* group with increasing polyamine concentration suggest that polyamines may be used by these microorganisms as growth factors. Polyamines may be the preferred substrate or they may stimulate proliferation and differentiation of mucosa cells in specific environments which are beneficial for these bacterial groups.

5. Conclusions

Changes in lifestyle during recent decades, including nutritional habits of nursing mothers, may influence breast milk composition, duration of breastfeeding and the physiological properties of some bioactive components present in breast milk. Surprisingly, little attention has been paid to the role of breastfeeding vs. formula feeding on the functional development of the digestive tract. Our results demonstrate the potential effect of polyamines on intestinal microbial composition with a resulting impact on health; such an effect should be further studied in human infants.

Taken together, enrichment of IF with polyamines influences microbial colonization patterns in mice, promoting similar microbiota to that found in mice with normal lactation. We suggest that the phenomenon could be related to that seen in healthy breastfed human infants [33]. Further studies are warranted to verify this phenomenon and the correlation with other factors associated with health status such as mucosal permeability to macromolecules or short-chain fatty acid production.

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