Food and Chemical Toxicology 60 (2013) 116–122

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Dietary supplementation of *Bifidobacterium longum* strain AH1206 increases its cecal abundance and elevates intestinal interleukin-10 expression in the neonatal piglet

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ARTICLE INFO

Article history:
Received 17 April 2013
Accepted 9 July 2013
Available online 18 July 2013

Keywords:
Bifidobacteria
Probiotic
Infant
Intestinal health
Immune system
IL-10

ABSTRACT

Intestinal microbiota of infants differ in response to gestational age, delivery mode and feeding regimen. Dietary supplementation of probiotic bacteria is one method of promoting healthy populations. We examined the impact of a novel probiotic strain of *Bifidobacterium longum* (AH1206) on the health, growth and development of neonatal pigs as a model for infants. Day-old pigs were fed milk-based formula containing AH1206 at 0, 10^9, or 10^{11} CFU/d for 18 d ($n = 10$/treatment). Differences were not detected in growth, organ weights or body temperatures ($P > 0.1$); however pigs fed the high dose showed a small (2%) reduction in feed intake. Bacterial translocation was not affected as indicated by total anaerobic and aerobic counts (CFU) in samples of spleen, liver and mesenteric lymph nodes ($P > 0.1$). Feeding AH1206 had no effects on fecal consistency, but increased the density of *B. longum* in the cecum. Ileal TNF expression tended to increase ($P = 0.08$) while IL-10 expression increased linearly ($P = 0.01$) with supplementation. Based upon findings in the suckling piglet model, we suggest that dietary supplementation with *B. longum* (AH1206) may be safe for human infants based on a lack of growth, development or deleterious immune-related effects observed in piglets.

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1. Introduction

Infants are born with an essentially sterile gut and colonization starts immediately during and after delivery (Mackie et al., 1999; Wang et al., 2013). The composition of the intestinal microbiota can be modulated by administration of live bacteria directly and a possible way to provide the necessary microbial organisms to formula-fed infants would be through the addition of well selected endogenous intestinal strains isolated from healthy, breast-fed infants. Bifidobacteria dominate the GI microbiome of breast-fed infants within the first two weeks of life, while formula-fed infants develop a more diverse flora, with *Bacteriodes* sp. equaling the number of bifidobacteria (Harmsen et al., 2000). Commensal bacteria associated with breast milk, such as bifidobacteria, help maintain healthy gastrointestinal mucosal surfaces (Ismail and Hooper, 2005) and play a critical role in stimulating maturation of the immune system (Gronlund et al., 2000). *Bifidobacterium longum* is part of the commensal bacterial community and inoculation of the infant intestine can occur directly through breast milk which may explain the differences in microflora between breast-fed and formula-fed infants (Martin et al., 2009). In order to more closely approximate both nutritional and functional properties of human milk, efforts are under way to identify novel ingredients to more closely align the bacterial community development of formula-fed infants to that of their breast-fed counterparts. To that end, supplementation of probiotic bifidobacteria strains is hypothesized to modify the bacterial community of formula-fed infants to be similar to that of breast-fed infants.

Probiotics have been defined as "living microorganisms that, on ingestion in sufficient numbers, exert health benefits beyond basic nutrition" (Weng and Walker, 2006). In pediatric populations, the administration of specific probiotic bacteria may reduce the incidence and duration of diarrhea (Weizman et al., 2005), shorten

Abbreviations: AH1206, *B. longum* AH1206; GI, gastrointestinal; IEL, intraepithelial lymphocytes; MPO, myeloperoxidase; NB, newborn; SR, sow-reared; TMB, tetramethylbenzidine.

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0278-6915/$ - see front matter Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.fct.2013.07.020

the duration of rotavirus diarrhea (Szymanski et al., 2006), reduce the incidence of atopic eczema in high risk children (Kalliomaki et al., 2001) and reduce the risk of necrotizing enterocolitis and overall mortality in preterm, very low birth weight infants (Deshpande et al., 2007). Reductions of duration and incidence of similar diseases have been observed in breast-fed infants (Howie et al., 1990).

The gastrointestinal immune system is in constant contact with antigens and provides both protection from infection and tolerance. In a murine neonatal model, AH1206 was shown to induce Foxp3$^+$ T regulatory cells, which is associated with protection from respiratory and oral allergy (Lyons et al., 2010). At the systemic level, splenocytes isolated from AH1206-fed mice had significantly greater release of the anti-inflammatory cytokine, IL-10, as compared to placebo fed controls (Lyons et al., 2010) indicating AH1206 may suppress aberrant activation of both the Th1 and Th2 response to promote the development of immunological tolerance. The use of novel ingredients to infant formula, including probiotic *Lactobacillus* and *Bifidobacterium* isolates depends in part on the establishment of a thorough safety profile (Carroll, 2004). Because the GI tract is the target site of probiotic activity and the process of GI maturation in neonatal piglets is closer to that of human infants compared to other common laboratory animal models, neonatal piglets were selected as a model to evaluate the safety and immunological impact of a new probiotic *Bifidobacterium* strain intended for possible use in infant formula. It is therefore proposed that supplementing infant formula with the novel probiotic strain *B. longum* AH1206 will lead to the establishment of this strain within the formula-fed piglet intestine. This study was conducted to determine the safety and efficacy of the novel bifidobacteria strain *B. longum* AH1206 and identify changes in intestinal immune status associated with supplementation.

2. Materials and methods

2.1. Animals and experimental diets

All animal procedures were approved by the Institutional Animal Care and Use Committee of North Carolina State University. Colostrum fed composite crossbred piglets (genetics derived from Pig Improvement Company, Hendersonville, TN) were acquired from the North Carolina State University Swine Education Unit within 12–24 h of birth and housed in an environmentally controlled room at Laboratory of Developmental Nutrition at North Carolina State University. All pigs were injected with iron dextran and penicillin prior to arrival at the laboratory. Piglets were obtained from six litters and were randomly assigned to one of three treatments and two reference groups (newborn, day 0 controls (NB) and sow-reared (SR)) based on body weight (n = 10/treatment). Sows were fed a common corn-and soybean-meal-based diet. Newborn pigs were from the same litters as treatment pigs and served as a control of baseline parameters so that progression of gastrointestinal development could be observed. Dietary treatments included the basal diet (Milk Specialties Global, Eden Prairie MN) and the basal diet supplemented with 10^9 or 10^{11} CFU per day of *B. longum* AH1206. Dietary treatments (milk formulas) were fed through a gravity flow system for accurate measurement of formula consumption (Mathews et al., 2002). The basal dry milk formula was designed as a sow milk replacer and is detailed in Table 1. Once the formula was reconstituted with water it was stored at 4 °C for a maximum of 48 h before use. The lyophilized AH1206 probiotic isolated from infant fecal samples (Lyons et al., 2010) was obtained from the Alimentary Health Ltd (Cork, Ireland) culture collection and stored at 4 °C in individual packets. Once daily, 10^9 or 10^{11} CFU AH1206 were added to the reconstituted formulas and offered to the pigs. For 2 d prior to starting the probiotic treatments, animals were fed the basal diet including antibiotics (Gentamycin sulfate) and treated with penicillin daily. After treatments were started, antibiotic treatment was discontinued. Newborn (NB) piglets were sampled after the 2 d of acclimation.

2.2. Animal feeding and management

Fresh formula was provided 3 times per day (0800, 1400 and 2100) and stored under refrigeration until fed as previously described (Herfel et al., 2011). Diets were reconstituted at 180 g dry diet in 1000 mL of water. The probiotic was added to the diet during the 1400 feeding period. Viability of the probiotic culture was confirmed by the manufacturer, and packets of the lyophilized probiotic were stored at 4 °C prior to the addition to the milk. Pig weights and feed intakes were recorded daily. Stool consistency was evaluated daily and rated on a 1 to 5 scale with 1 representing well-formed stools and 5 representing watery stool with no form. Sow-reared pigs were maintained at a separate location. Rectal temperatures were determined for all treatments on days 0, 7, 14 and 18. Pigs were euthanized on day 0 (NB pigs) and day 18 (formula fed and sow-reared pigs) by AVMA-approved electrocution and exsanguination and tissues were collected immediately.

Table 1
Composition of basal formula.

Ingredient	g/100 g of diet
Whey	29.0
Edible lard	19.4
Whey protein concentrate	18.2
Delactosed whey	18.1
Sodium caseinate	11.3
Dicalcium phosphate	2.0
Other[a]	2.0
Total	100.0
Calculated nutrients	
Energy (kcal/g)	4.9
Crude protein (%)	27.7
Crude fat (%)	18.0
Carbohydrate (%)	54.3

[a] Microingredients, g/kg diet: mineral premix, 5.0; D/L methionine, 4.9; potassium sorbate, 4.5; calcium chloride, 3.3; emulsifier, 2.2; lecithin, 1.9 sodium hexametaphosphate, 1.8; flow agent, 1.4; vitamin premix, 1.1; flavor additive, 0.3; antioxidant, 0.05; Milk Specialties Global, Eden Prairie, MN 55344.

2.3. Sample collection

Prior to euthanasia blood samples were collected followed by plasma and serum separation and storage at −20 °C. Clinical blood parameter measurements (VetScreen and Whole Blood Analysis) were conducted by Antech Diagnostic Laboratories (ADL; Cary, NC). A urine sample also was collected via bladder puncture for urinalysis by ADL. The large and small intestines were then isolated and mucosal and histological tissue samples were collected proximal to the ileocecal junction. Digesta were collected from the remaining portion of the ileum, the entire colon and cecum. Digesta from each site were sub-sampled for various analyses. The pH of digesta was determined immediately, prior to freezing. Organ weights were recorded for the liver, gallbladder, kidney, spleen, pancreas, cecum, colon, thymus, lung, heart, brain and eye. Ileal mucosa was collected, frozen in liquid nitrogen and stored at −80 °C for later analysis of cytokine message and enzyme activity.

2.4. Bacteria translocation

The surface of the liver, spleen and mesenteric lymph nodes were swabbed with an anaerobic and aerobic transport swab in duplicate using aseptic technique, and swabs were placed on ice. Swabs were then streaked in duplicate on either anaerobic blood agar (total anaerobic bacteria) and incubated in anaerobic conditions or nutrient agar for aerobic bacteria. Immediately after plating, anaerobic blood agar plates were placed in an anaerobic chamber with 5% CO_2, 5% H_2, 90% N_2 atmosphere and incubated for 48 h at 37 °C. The nutrient agar plates were incubated aerobically ay 37 °C 48 h. Organs were considered positive for translocation if CFU were higher than the threshold of 30 colonies per plate (Barrat et al., 2008).

2.5. Bacterial determination via quantitative PCR

Intestinal bifidobacteria were quantified using methods and primers described by Matsuki and colleagues (2004). The primers were designed to detect total *B. longum* rather than the AH1206 strain, specifically. To determine cell equivalents/g digesta a standard curve was prepared. In duplicate, *B. longum* was inoculated in reinforced Clostridial medium and incubated at 37 °C. Every 4 h media were collected and the optical density read at 600 nm. At each time point 5 dilutions were made with 0.1 × MRS broth containing 0.05% cysteine. Dilutions were plated in duplicate on RCA in an anaerobic chamber with 5% CO_2, 5% H_2, 90% N_2 atmosphere and incubated for 48 h at 37 °C. An additional subsample was pelleted via centrifugation and frozen at −80 °C to serve as a cell equivalent reference. DNA was isolated following the manufacturer's instructions (Qiagen DIAamp DNA Stool Mini Kit, Valencia, CA). Amplification was carried out in a total volume of 25 μL containing 1X iQ SYBR Green Supermix (BioRad Laboratories, Hercules, CA), forward and reverse primers (200 nM each) and 5 μL of the isolated DNA (BioRad Laboratories, Hercules, CA). At the end of the PCR, melt curve analysis was conducted to validate the specificity of the primers. All determinations were performed in at least duplicate.