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Dissecting the role of milk components on gut microbiota composition

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The composition of human milk is tailored to contribute to the development of the gastrointestinal (GI) tract of newborns and infants. Importantly, human milk contains the antimicrobial compounds lysozyme and lactoferrin that are thought to contribute to the formation of a health-promoting microbiota. As these protective factors are lacking in the milk of dairy animals, we genetically engineered goats expressing human lysozyme in their milk and have recently reported a new animal model to dissect out the role of milk components on gut microbiota formation. Using the pig as a more human-relevant animal model, we demonstrated that consumption of lysozyme-rich milk enriched the abundance of bacteria associated with GI health and decreased those associated with disease, much like human milk. This work demonstrated that the pig is a valid animal model for gut microbiome studies on the effects of dietary components on microbiota composition, host-microbe interactions and state of the intestine.

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Protective Properties of Milk

The microbial population of the GI tract plays a key role in maintenance of nutrition, host defense and immune development. Therefore, alteration of the GI microbiota can have profound effects. Establishment of GI microbiota begins just after birth and is influenced by the presence of non-specific immunologic components present in human milk, including lysozyme and lactoferrin. A number of lines of evidence suggest that these antimicrobial components contribute in several ways to the health and well-being of breast-fed infants including defense against infection by pathogenic organisms, the stimulation of a beneficial gut microbiota, development and maturation of the intestinal tract, and by acting as anti-inflammatory agents.[1,2] The development of a healthy GI tract and mucosal immune system contributes to the recognized benefits that breast-fed infants have over formula-fed infants including lower incidences of acute and chronic illnesses both early and later in life.[3] One of the most remarkable differences found between breast-fed and formula-fed infants is the composition of their respective intestinal microbiota populations. The fecal microbiota of formula-fed infants is more complex with coliforms, enterococci, bacteroides, clostridia and streptococci all being prevalent, while breast-fed infants tend to have a rather simple microbiota population, consisting primarily of lactobacilli and staphylococci along with bifidobacteria.[4,5] The differential composition of GI microbiota in infants fed formula, which lacks key human milk components including oligosaccharides, lactoferrin and lysozyme, points to the presence of these factors as being able to promote the formation of a beneficial intestinal microbiota. The determination of the individual role of these components on the formation of gut microbiota is important as a beneficial GI microbiota can help confer a number of positive attributes including protection against diarrhea, inflammation and GI illness. Knowledge of how milk and other dietary components influence GI microbiota could lead to the identification of specific protective factors that promote GI and overall health.

Lysozyme is a ubiquitous antimicrobial molecule that exists naturally in avian egg whites and mammalian secretions such as tears, saliva and milk[6] as part of the natural defense mechanism against bacterial infection. Lysozyme specifically catalyzes the cleavage of the glycosidic linkage between the C-1 of N-acetylmuramic acid and the C-4 of N-acetylglucoseamine that make up the peptidoglycan component of bacterial cell walls. Cleavage of the peptidoglycan layer by lysozyme causes leakage of the cell's interior components and results in cell lysis. Both Gram-positive and Gram-negative bacteria are susceptible to lysozyme.[7] Gram-positive bacteria have a thicker peptidoglycan layer making them more resistant to lysozyme than Gram-negative bacteria where once the protective outer membrane is disrupted, lyszoyme has access to a thin layer of peptidoglycan.

In contrast to human milk where levels of lysozyme start low and then increase and maintain a high level throughout lactation, the opposite is true in the milk of dairy animals (goats and cows). Lysozyme levels are high only during an udder infection and at times when the animal is most susceptible to infection (birth and weaning) when lysozyme is recruited to the mammary gland to help fight the infection, but are normally maintained at 1,600- to 3,000-fold lower levels than human milk throughout the course of lactation.[8] Due to this physiological occurrence, it is not possible to select dairy animals naturally producing high levels of lysozyme to propagate. We have thus generated a line of transgenic dairy goats expressing human lysozyme (hLZ) in their milk at levels of 270 ± 84 μg/ml.[9,10] This represents a 1,000-fold increase over the mean level of lysozyme normally present in goat milk and is approximately 68% of that normally found in human milk (400 μg/ml). Further, the lysozyme was found to be active in vitro against bacterial isolates responsible for disease and milk spoilage.[11] The percentage of milk yield that represents total fat and protein fell in the same range as the means for the non-transgenic control dairy goat herd,[10] indicating that expression of the transgene did not disrupt the gross composition of milk. Furthermore, basic functions, such as growth and reproduction of the transgenic animals themselves, were not adversely impacted by either the presence or expression of the transgene, or by consumption of the hLZ-containing milk.[12] These transgenic animals were produced with the intent to improve udder health, milk safety and longevity and the nutritional quality and health benefits of milk when consumed. We developed a pig model to test the health benefits of this lysozyme-rich milk as well as to explore milk components that modulate gut microbiota.

Animal Models of GI Microbiota Studies

Current animal models for research on GI microbiota consist mainly of laboratory species such as the mouse and more recently, zebrafish.[13] While these are excellent model organisms for toxicity studies and elucidating mechanisms of actions of pathways, genes and proteins, extrapolation to human health is not always direct. A model organism, such as the pig, may provide a more human-relevant model for future studies of dietary interactions with the microbiome. Pigs represent a monogastric animal with GI anatomy, function, and metabolic regulation similar to humans. The use of pigs as a relevant human medical model is well documented[14] and in many cases is a better model animal for nutritional studies and the study of human illnesses, including those of the intestine as the pig has 90% gut homology with humans.[15] The development, anatomy, physiology, metabolism, genetics and immune system of the pig GI tract is more similar to humans than are those of the mouse.[16,17] Furthermore, genetically, the pig is closer to the human than the mouse[18,19] and sequence and EST libraries exist to allow for molecular-based analyses.[20] Importantly for microbiota research, pigs and humans share similarities in GI microbial diversity.[21] Coupled with the availability of sequence information, concurrent gut microbiota, transcriptome and metabolome analyses can be performed to complete the picture of how GI microbiota impacts host-microbe interactions. These types of global analyses will improve the understanding of the role of GI microbiota on host defense and allow for an increased understanding of the pathway interactions between gut microbes and the host. The pig also enables invasive studies along the length of the GI tract which cannot be easily performed in humans. In addition, the development of pig models of human GI diseases such as inflammatory bowel diseases (IBD) would be useful in assessing potential therapies and causes of IBD and the role the GI microbiota plays in mitigating these conditions. While mice and zebrafish are readily available and easy to work with, without much more effort similar work also can be conducted in the pig. Performing gut microbiota research in a more human-relevant model such as the pig would add to the scientific knowledge and close the gap to one step closer to the human.

Lysozyme Alone in Milk Can Alter GI Microbiota

In a recent paper in *Applied and Environmental Microbiology* we reported the use of a pig model which demonstrated the ability of lysozyme-rich milk to modulate fecal microbiota composition much like human milk.[22] Six week old pigs were fed 250 ml pasteurized milk from either hLZ transgenic or non-transgenic control goats twice daily for a period of 14 d. Fecal samples were collected at various time points and the microbial diversity was determined using 16S rRNA gene sequencing of clone libraries and the G2 Phylochip.[23] Through the use of western blots, 2D gels, and mass spectroscopy, it was shown that the protein composition of the milk from transgenic and control animals differed only in the presence of human lysozyme, thus all effects could be attributed to the action of lysozyme. Microbial populations of pig feces prior to and during milk supplementation were similar to human feces with *Bacteroidetes* and *Firmicutes* representing the major phyla.[22] These observations contribute to the evidence that the pig is an acceptable model for studying the GI microbiome and consequences of microbiota manipulation.

Both methods (sequencing of clone libraries and the Phylochip) gave similar results with the Phylochip detecting substantially more operational taxonomic units (OTUs) or number of phylotypes as

defined by distinct 16S rRNA sequences. Each feeding group (control milk or hLZ milk) had a similar total community population with the populations being distinct depending on milk type fed. Compared with control milk-fed pigs, the microbiota of hLZ-fed pigs more closely resembled that of human infants being breast-fed. Levels of Firmicutes (mainly *Clostridia* spp.) declined while Bacteroidetes increased over time in response to consumption of lysozyme-rich milk. This same trend was found in a study comparing the fecal microbiota of breast-fed and formula-fed infants,[24] again indicating the relevance of the pig as an animal model for microbiota studies with respect to human health. These results also highlight the significant role that lysozyme in milk is playing on the composition of the microbiota. Also like breast-fed infants, the consumption of hLZ milk resulted in the enrichment of Bifidobacteriacea and Lactobacillacea, both biomarkers of increased gut and host health.[25] These beneficial changes were accompanied by the reduction of *Clostridia* spp. and *Streptococcaceae*, common components of the fecal microbiota of formula-fed infants, as well as decreased levels of disease-related bacteria including *Mycobacteriaceae* and Campylobacterales. These results indicate that the feeding of hLZ milk results in the reproducible modulation of GI bacteria, thus making this a robust and biologically relevant model system in which to study the impact of GI microbiota changes. Further work investigating the impact of beneficial microbe enrichment caused by lysozyme and other milk components will give insight on how individual components of the diet can selectively alter GI microbiota and the subsequent impact of the resulting biota on host-microbe interactions eventually leading to the identification of key community members and their role in the gut.

Other Impacts of Lysozyme-Rich Milk at the Level of the Intestine

Previous work in pigs has demonstrated that consumption of pasteurized milk from hLZ transgenic goats resulted in changes at the level of the intestine that are indicative of improved GI health.[26] Animals receiving hLZ milk tended to have longer villi and had a significantly thinner lamina propria in the duodenum demonstrating increased gut absorptive area. The number of intraepithelial lymphyocytes was significantly increased in the ileum of hLZ-fed animals where they also tended to have an increase in the number of mucin-producing goblet cells, indicating increased protection of the intestinal epithelium. The expression of key cytokine genes in intestinal segments was analyzed by qPCR and demonstrated that consumption of hLZ milk significantly increased the expression of the anti-inflammatory cytokine TGFβ in the ileum, again indicating a healthier gut.[26] Expression of the pro-inflammatory cytokines TNFα and IL-8 was not significantly different between control and hLZ-fed animals, indicating that an inflammatory response is not induced upon consumption of hLZ milk. In addition, standard CBC analysis demonstrated that no allergic response was occurring upon consumption of hLZ milk.[27] Finally, serum from the pigs was subjected to a metabolomic analysis.[28] A total of 234 metabolites were quantified with levels of 22 metabolites significantly different in pigs fed hLZ milk compared with pigs that received control milk. These differences could be broken down into effects of bacteria, increased growth, healthier GI tract and modulation of immune system with the direction of changes indicative of a healthier gut. These results demonstrate that hLZ milk is capable of altering the state of the intestine either directly and/or through changes in the composition of the GI microbiota.

Extending the Pig Model to Test Effects of Other Milk Components

Recently we have extended this work to assess the effects of human lactoferrin (hLF) on the GI tract using our pig model. Lactoferrin is an iron binding protein that, like lysozyme, is present at high levels in human milk but not in the milk of dairy animals. Lactoferrin is part of the host defense system, acting in both an antimicrobial and immunomodulatory fashion.[1] Pasteurized milk from transgenic cows producing hLF within the range found in human milk (1.5–2.0 g/L)[29] was fed to six week old pigs for a period of two weeks followed by complete blood count (CBC) analysis and evaluation of GI architecture and cytokine expression.[30] Pigs fed hLF milk had an increased gut surface area accounted for by significantly taller villi, deeper crypts and thinner lamina propria in the ileum, as seen with pigs fed lysozyme-rich milk but to a greater extent. Furthermore the lactoferrin-fed animals had a decreased neutrophil to lymphocyte ratio, an indicator of decreased systemic inflammation, but no differences in pro- and anti-cytokine expression in the intestinal tissue was observed. Recent work demonstrated that the glycosylation of lactoferrin is also important in modulating pathogen adhesion in vitro,[31] suggesting that the glycan component in addition to the iron binding capacity of lactoferrin plays a role in gut flora development. This work further validates the pig model for GI tract studies and indicates that lactoferrin-rich milk also has the potential to induce positive changes in the GI tract. The effect of hLF milk on the microbial populations of the GI tract remains to be determined.

Conclusions

The mechanisms by which changes in enteric microbiota impact intestinal and systemic health remain poorly defined. We developed a novel model with which it is possible to look at the effects of GI microbiota changes on disease. Milk from the hLZ transgenic goats is able to reproducibly shift the composition of the GI microbiota to contain an increased proportion of microbes that are considered biomarkers of good GI health. This model will allow for the further evaluation of the complex interactions between nutrient (lysozyme or lactoferrin), host and bacterial populations and can be extrapolated to other milk or dietary components. Furthermore, this model can be used to evaluate the potential of lysozyme-rich milk to improve the state of the intestine to better combat disease. For instance, each year more than 1.5 million children die worldwide as a result of common GI tract infections.[32] Oral rehydration solution and breast-feeding are recommended

as treatment with breast-fed children having a documented reduction in diarrhea episodes and faster recovery time. This improvement can in part be attributed to the antimicrobial actions of human milk proteins, such as lysozyme and lactoferrin. The ready availability of livestock milk containing human milk protective proteins that can act to improve intestinal health has implications not only for common bacterial-induced diarrheal illnesses but also for those suffering from IBD and malnourishment. Much work remains to be done to elucidate the effects individual milk components and milk as a whole have on the composition of the microbiome and GI tract health in both healthy and diseased states. The pig as a model animal for GI research is poised to help answer these questions and others related to the nutritional modulation of gut microbiota.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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