

Consumption of Lysozyme-Rich Milk Can Alter Microbial Fecal Populations

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Human milk contains antimicrobial factors such as lysozyme and lactoferrin that are thought to contribute to the development of an intestinal microbiota beneficial to host health. However, these factors are lacking in the milk of dairy animals. Here we report the establishment of an animal model to allow the dissection of the role of milk components in gut microbiota modulation and subsequent changes in overall and intestinal health. Using milk from transgenic goats expressing human lysozyme at 68%, the level found in human milk and young pigs as feeding subjects, the fecal microbiota was analyzed over time using 16S rRNA gene sequencing and the G2 Phylochip. The two methods yielded similar results, with the G2 Phylochip giving more comprehensive information by detecting more OTUs. Total community populations remained similar within the feeding groups, and community member diversity was changed significantly upon consumption of lysozyme milk. Levels of *Firmicutes* (*Clostridia*) declined whereas those of *Bacteroidetes* increased over time in response to the consumption of lysozyme-rich milk. The proportions of these major phyla were significantly different ($P < 0.05$) from the proportions seen with control-fed animals after 14 days of feeding. Within phyla, the abundance of bacteria associated with gut health (*Bifidobacteriaceae* and *Lactobacillaceae*) increased and the abundance of those associated with disease (*Mycobacteriaceae*, *Streptococcaceae*, *Campylobacterales*) decreased with consumption of lysozyme milk. This study demonstrated that a single component of the diet with bioactivity changed the gut microbiome composition. Additionally, this model enabled the direct examination of the impact of lysozyme on beneficial microbe enrichment versus detrimental microbe reduction in the gut microbiome community.

Human milk not only provides the newborn with all the nutrition it needs to grow and develop but also provides factors that promote health and combat infection. One of the main non-specific host defense factors in human milk is lysozyme. Lysozyme is a naturally occurring antimicrobial enzyme found in the tears, saliva, and milk of all mammals that lyses a specific link in the peptidoglycan layer of bacterial cell walls, resulting in cell lysis (33). Along with lactoferrin and secretory IgA, lysozyme contributes to the nonspecific immunity associated with milk consumption. A number of epidemiological studies have documented the benefits of human milk, including advantages in general health, growth, and development and protection against a number of acute and chronic diseases in infancy and beyond (16, 22). In addition to its role in host defense, human milk also helps with the establishment of a beneficial gut microbiota. Breast-fed infants have been shown to have a more healthy and simple gut microbiota consisting mainly of bifidobacteria, lactobacilli, and staphylococci, while formula-fed infants have a more complex microbiota, with coliforms, clostridia, enterococci, streptococci, and bacteroides all being prevalent in the gut (1, 15, 34). In addition to human milk oligosaccharides and other bioactive compounds, another reason for the growth of fewer facultative anaerobes in breast-fed infants is thought to be the presence of antimicrobial factors such as lysozyme in human milk (18, 34).

The composition and function of the intestinal microbiota are just beginning to be defined and ascertained, but it is widely accepted that it plays a role in both health and disease (41). Due to the purported role of human milk in gut microbiota community formation, a source of milk rich in lysozyme may shift the microbial population ratios in the gut during milk consumption toward those microbes associated with health for the host. While cow and goat milk is readily available, it is low in key health-promoting antimicrobial components for humans. Human milk contains 400

$\mu\text{g/ml}$ lysozyme, while cow milk and goat milk contain 3,000 (0.13 $\mu\text{g/ml}$) and 1,600 (0.25 $\mu\text{g/ml}$) times less lysozyme, respectively (8). We have developed transgenic dairy goats that express human lysozyme (HLZ) in their milk at 68% the level normally found in human milk (29) with the goal of incorporating the beneficial protective properties of human milk into readily available livestock milk in order to promote the intestinal and overall health of people of all ages. We previously demonstrated that consumption of milk from HLZ-transgenic goats by animal models results in the modulation of *Escherichia coli* and total coliform levels in the small intestine as determined using standard culture techniques (30), resistance to intestinal colonization by pathogenic *E. coli* (5), and histological and cytokine changes coupled with increases in serum metabolite makers indicative of improved gastrointestinal (GI) health (6, 10).

In order to determine if HLZ-rich milk can modulate gut microbiota composition, this study conducted feeding trials in young pigs followed by a more in-depth fecal microbiota assessment using 16S rRNA clone libraries and the G2 Phylochip. The pig was selected as a model organism since it represents a monogastric animal with GI anatomy, function, and metabolic regulation similar to those of humans (2). The use of pigs as a relevant

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human medical model is well documented (27). Furthermore, pigs and humans share similarities in GI microbial diversity (20) and pig studies allow more invasive sampling, induction of disease states, and a variety of nutritional intervention approaches. This study found that HLZ milk modulated GI microbiota by increasing the ratio of beneficial bacteria and decreasing disease-causing microbe numbers.

MATERIALS AND METHODS

Milk and milk analysis. Production and characterization of the HLZ-transgenic line has been previously described (28, 29). This line of transgenic goats produces active HLZ in their milk at an average level of 270 µg/ml without disrupting the gross composition of the milk in terms of total fat and protein content (29). For the feeding to pigs, milk was collected by machine at the morning and evening milkings from HLZ-transgenic and nontransgenic control goats from the University of California (UC) Davis dairy goat herd. Milk was pooled into respective containers, pasteurized to 74°C, and stored at 4°C prior to feeding to animals. Milk was used for 1 week before being discarded and replaced with a new batch. Each batch of milk was analyzed by Western blotting to confirm the presence and quantity of HLZ. Milk was also collected from a total of six individual HLZ-transgenic and equal numbers of age- and breed-matched nontransgenic control animals for a more in-depth analysis using two-dimensional (2-D) gel analysis coupled with mass spectrometry. Milk was collected at peak lactation (2 months) from two transgenic animals each in their 1st, 2nd, and 3rd lactations and equal numbers of breed- and parity-matched nontransgenic controls. A total of 200 µg of protein from each milk sample was subjected to 2-D gel analysis followed by matrix-assisted laser desorption ionization–time of flight/time of flight (MALDI-TOF/TOF) mass spectrometry as previously described (32). Briefly, separation in the first dimension by isoelectric point was carried out using Immobiline DryStrips (GE Healthcare, Piscataway, NJ) (pH 3 to 5.6; 11 cm in length) followed by separation in the second dimension by size on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (Protean Xi; Bio-Rad, Hercules, CA). Gels were then stained in Coomassie blue and scanned, and proteins were quantified using the All-to-One warping strategy with Delta 2D gel analysis software (Decodon GmbH, Greifswald, Germany). A total of 29 individual spots were then extracted from each of three gels containing milk from transgenic goats in their 2nd and 3rd lactation and their three corresponding control gels and processed using a Montage In-Gel Digest Zip kit (Millipore, Billerica, MA) followed by MALDI-TOF/TOF mass spectrometry. The same 29 spots were chosen on each of the six gels. Protein identification and annotation were carried out using GPS Explorer software with the Mascot search algorithm and DeNovo Explorer modules included in the 4700 Explorer software (Applied Biosystems, Foster City, CA). All animals were housed and cared for under AAALAC-approved conditions.

Feeding trials. Eight crossbred, specific-pathogen-free Yorkshire full-sibling male pigs from the UC Davis Swine Facility were weaned at 21 days of age and housed together, receiving the same treatment and diet until 8 weeks of age, at which time they were placed into groups of two in connected environmental chambers. All pigs had *ad libitum* access to a standard ration of dry feed (UC Davis Pig Starter Diet; Associated Feed, Turlock, CA) and water throughout the trial. In addition to the standard diet, each pen received 500 ml of pasteurized nontransgenic control goat milk twice daily delivered through a lixit container for a period of 2 days in order for the animals to become accustomed to consuming milk. Each animal was then placed into an individual pen, with four animals receiving pasteurized milk from nontransgenic control goats and four receiving pasteurized milk from HLZ-transgenic goats for a period of 14 days. Pigs that were cohoused for the adjustment period were given the same type of milk. Each animal was dosed with 250 ml of its respective milk allotment twice daily for 4 days, 300 ml twice daily for 3 days, and then 350 ml twice daily for the remaining 6 days. Fresh feces samples were collected from each pair of animals before any milk was given (day 1) and then from

individual animals at the end of the adjustment period and before the milk type was switched (day 3), 24 h after the start of HLZ milk administration (day 4), and then every other day for the remainder of the trial. After 14 days of milk dosing, the animals were subjected to necropsy and sections of the duodenum and ileum taken for total coliform and *E. coli* counts using Petrifilm count plates (3M, St. Paul, MN) as previously described (5).

16S rRNA gene sequencing. Fecal samples from days 1 (no milk), 3 (end of adjustment period), 6 (72 h on HLZ milk), and 17 (endpoint after 14 days of HLZ milk) were processed for sequence analysis. All feces samples were stored at 4°C for no longer than 4 days prior to bacterial DNA extraction using a QIAmp DNA stool kit with the protocol for pathogen detection (Qiagen, Valencia, CA). Primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1392R (5'-GACGGCGGTGTGTAC-3') were used to amplify the 16S rRNA gene sequence (21). PCRs were performed as recommended by Polz and Cavanaugh (39) to reduce bias in amplification. Briefly, PCR was performed in 50-µl reaction volumes containing 50 ng DNA and 1 µM each primer under the following conditions: 1 cycle of 95°C for 5 min, 20 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1.5 min, and 1 cycle of 10 min at 72°C. Resulting PCR products were ligated into StrataClone PCR cloning kit vector (Agilent Technologies, Santa Clara, CA) to create a library of clones for sequencing. A total of 192 clones per sample were grown in 96-well plates in LB freezing media, and DNA templates were prepared by rolling-circle amplification using a TempliPhi HT amplification kit (GE Healthcare, Piscataway, NJ) and then sequenced using primer 1392R and a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA).

Resulting DNA sequences were subjected to base-calling by PHRED and quality trimming and vector removal by LUCY and used for comparison to 16S rRNA gene sequences from the Ribosomal Database Project (release 10; <http://rdp.cme.msu.edu/>). Only sequences with unambiguous reads of greater than 400 bp were used. Clones with more than 97% sequence identity were defined as operational taxonomic units (OTUs) and assigned to a phylum using the Classifier software available at <http://rdp.cme.msu.edu/classifier/classifier.jsp> (49), which assigns an OTU sequence to a phylum using a naïve Bayesian rRNA classifier trained on the known type strain 16S sequences. Complete 16S rRNA libraries for each feeding group were compared to each other using the Student *t* test and the Library Compare software available at <http://rdp.cme.msu.edu/comparison/comp.jsp> (49), which estimates the likelihood that the frequency of membership in a given taxon is the same for the two libraries. The percentage of a phylum in one library was considered significantly different ($P < 0.05$) from the percentage in another library if the two statistical methods (Student's *t* test and Compare) were in agreement. As one control-fed pig was found to be an outlier via G2 Phylochip analysis, this pig was not used in the analysis of the 16S rRNA data.

G2 Phylochip. Analysis of fecal microbial diversity was also conducted on the endpoint samples (day 17) using the G2 Phylochip as described by Parnell et al. (37, 38). Briefly, the ribosomal 16S gene was amplified by PCR utilizing bacterium-specific primers (F [5'-AGAGTTTGATCCTGGCTCAG-3'] and R [5'-ACGGCTACCTTGTTAGCACTT-3']). Amplified and labeled DNA (4 µg) was hybridized to the G2 Phylochip for 16 h at 48°C, with rotation at 60 rpm. The chip was washed, stained, and scanned following the standard Affymetrix protocol at the Center for Integrated BioSystems, Utah State University (Logan, UT). Raw .cel files were normalized using RMA (17). The presence or absence of each OTU was determined using Phylotrac (www.phylotrac.org). An OTU was considered present if 90% of the probes in the OTU probe set had a ratio of PM/MM > 1.3. OTUs called present in at least one of the samples were included in further analysis. Hierarchical clustering of data was done using HCE 3.5 (42). Principal component analysis was done using TMEV 4.6.1 (40). SAM (46) was used to identify OTUs statistically significantly different between the control and treatment groups. Phylogenetic groups were created based on the phylum, class, order, and family of each OTU present on the Phylochip. Statistical overrepresentation of the OTUs due to the

TABLE 1 Identification and quantification of proteins in HLZ and control milk

Gel	Protein identification	Spot density (% vol)			SwissProt accession no.	Mascot score	Peptide count
		Control (<i>n</i> = 6)	HLZ (<i>n</i> = 6)	<i>P</i>			
ID32847	Serum albumin precursor	0.681 ± 0.133	0.728 ± 0.135	0.811	P14639	81	28
ID32983	β-Casein precursor	1.492 ± 0.367	1.357 ± 0.277	0.774	P11839	196	8
ID34457_N	β-Casein precursor	17.278 ± 0.979	17.192 ± 0.950	0.951	Q9TSI0	66	3
ID34461_N	β-Casein precursor	19.646 ± 1.461	20.338 ± 1.345	0.735	P11839	136	8
ID34468	α _{s1} -Casein precursor	4.001 ± 0.822	4.476 ± 0.897	0.704	P18626	161	8
ID34477	β-Casein precursor	5.829 ± 0.216	5.655 ± 0.509	0.759	Q9TSI0	176	5
ID34478	α _{s1} -Casein A short form	1.600 ± 0.426	1.342 ± 0.434	0.680	gi 999049	76	3
ID34484	β-Casein precursor	5.208 ± 0.504	5.152 ± 0.463	0.936	P33048	166	2
ID34485_N	β-Casein precursor	2.317 ± 0.539	2.423 ± 0.232	0.859	P11839	61	4
ID34488_N	β-Casein precursor	2.904 ± 0.205	2.975 ± 0.220	0.817	P33048	191	6
ID34504_N	κ-Casein precursor	1.642 ± 0.263	1.363 ± 0.165	0.390	P02670	211	4
ID34505	κ-Casein precursor	8.761 ± 0.464	9.148 ± 0.294	0.497	P02670	171	6
ID34516	α _{s1} -Casein precursor	0.715 ± 0.274	0.587 ± 0.228	0.727	P04653	151	10
ID34517	α _{s1} -Casein precursor	0.871 ± 0.277	1.083 ± 0.325	0.630	P18626	146	7
ID34530	α-Lactalbumin	12.180 ± 0.719	12.066 ± 0.692	0.911	P00712	86	2
ID46338_N	α _{s2} -Casein precursor	14.202 ± 0.753	14.198 ± 1.007	0.997	P04654	126	10
ID46339_N	α _{s2} -Casein precursor	9.162 ± 0.543	9.306 ± 0.660	0.870	P33049	111	14
ID46344_N	α _{s2} -Casein precursor	7.574 ± 0.413	7.393 ± 0.321	0.737	P04654	103	7
ID46345_N	α _{s2} -Casein precursor	2.099 ± 0.192	2.158 ± 0.283	0.866	P04654	91	13
ID46347_N	α _{s2} -Casein precursor	4.080 ± 0.388	4.110 ± 0.495	0.963	P04654	96	16
ID46351	α _{s2} -Casein precursor	4.507 ± 0.583	4.671 ± 0.426	0.825	P33049	121	8
ID46387	β-Lactoglobulin precursor	17.566 ± 1.001	18.033 ± 2.158	0.848	P02756	181	10
ID7607	α _{s2} -Casein precursor	1.422 ± 0.236	1.809 ± 0.285	0.320	P04654	116	12

treatment within specific phylogenetic groups was estimated using GSEA (44). 16S sequences for OTUs and family-wide consensus sequences were obtained from Greengenes (12). The 16S sequences were aligned using MUSCLE (14), maximum-likelihood trees were built using RAXML (43), and iTOL ver. 2.1.1 (24) was used to project log₂ ratios of data from the treatment versus control groups onto the 16S trees.

RESULTS

Milk analysis. The overall protein composition of milk from HLZ-transgenic goats was not statistically different from that of milk from nontransgenic control goats. A total of 136 protein spots were analyzed on 2-D gels, and the numbers of proteins and their locations and intensities were not significantly different between transgenic and control samples (Table 1; see also Fig. S1 in the supplemental material). Furthermore, 23 of the 29 spots chosen for mass spectrometry analysis were identified and all corresponded to proteins normally found in milk (Table 1). Pigs consuming HLZ milk for 14 days had significantly lower numbers of coliforms in the duodenum than did control-fed pigs (*P* = 0.019; Table 2). The numbers of coliforms and *E. coli* in the ileum were lower but not significantly different in HLZ-fed pigs (*P* = 0.487 and *P* = 0.332, respectively).

16S rRNA clone sequencing analysis. The fecal microbial profile determined using 16S rRNA gene sequencing showed that all animals started with similar fecal microbial populations, as no significant differences in phyla among animals at day 1 and day 3 were observed (Fig. 1). The fecal microbial profile changed significantly after 14 days of HLZ milk consumption (Table 3). Animals receiving HLZ milk had an overrepresentation of *Bacteroidetes* approaching significance (*P* = 0.07) and significant underrepresentation of *Firmicutes* (*P* = 0.038) compared to animals receiving control goat milk. Within the *Bacteroidetes*, members of the order

Bacteroidales were present in a significantly greater proportion in HLZ-fed animals (*P* = 0.035), with a significantly higher representation from the genera *Paraprevotella* and *Parabacteroides* (*P* = 0.033; Table 4).

The consumption of control milk during the adjustment period promoted the growth of members of *Proteobacteria* (*P* = 0.0063), specifically, of the genus *Succinivibrio* (*P* < 0.0048). Over time, levels of *Bacteroidetes* steadily increased and were accompanied by a steady decrease in levels of *Firmicutes* in HLZ-fed pigs whereas these populations fluctuated in control-fed pigs (Fig. 1A). There were significant differences in the levels of *Firmicutes* in control and HLZ-fed pigs by day 6 (*P* = 0.013), and they remained significantly different at day 17 (*P* = 0.038). On day 6, HLZ-fed pigs also had significantly fewer *Tenericutes* (genus *Anaeroplasmata*) than did control-fed pigs (*P* < 0.001, Table 4). Within the *Firmicutes*, levels of the class *Clostridia* gradually decreased over time in HLZ-fed pigs and were significantly different from those in con-

TABLE 2 Total coliform and *E. coli* counts in intestinal segments at day 17

Segment and organism(s)	log CFU/g ± SD for indicated pig group	
	Control fed (<i>n</i> = 3)	HLZ fed (<i>n</i> = 4)
Duodenum		
Coliforms	1.93 ± 0.81	0.25 ± 0.5 ^a
<i>E. coli</i>	0	0
Ileum		
Coliforms	3.66 ± 1.46	3.01 ± 0.82
<i>E. coli</i>	3.66 ± 1.46	2.36 ± 1.65

^a HLZ-fed pigs significantly different from controls (*P* = 0.019).

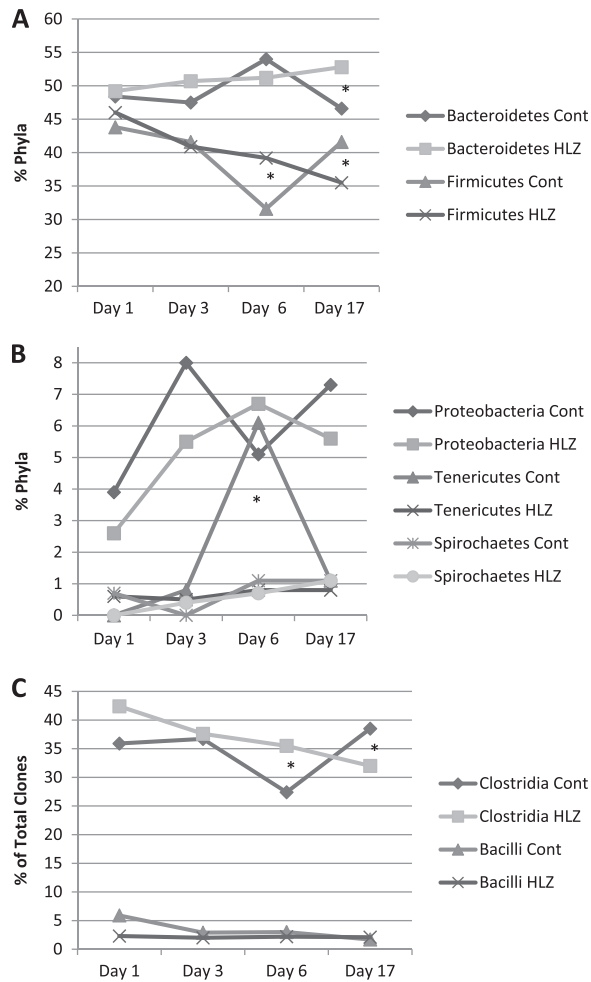


FIG 1 Changes in phyla over time. Percentages of clones assigned to (A) major phyla, (B) minor phyla, and (C) *Firmicutes* determined by 16S rRNA sequencing are shown. HLZ, pigs fed milk from HLZ-transgenic animals; Cont, pigs fed milk from nontransgenic control animals. *, significantly different ($P < 0.05$).

control-fed pigs at both day 6 ($P = 0.005$) and day 17 ($P = 0.045$), with more fluctuation in the control-fed animals (Fig. 1B and Table 4). There were significantly fewer bacteria from the *Subdoligranulum* and *Anaerovibrio* genera in HLZ-fed animals at day 17 (Table 4). Levels of *Bacilli* were steadily maintained in HLZ-fed animals and decreased over time in control-fed animals, although these differences were not statistically different ($P = 0.43$ at day 6 and $P = 0.682$ at day 17) (Fig. 1C).

G2 Phylochip analysis. All microbes and trends found in the clone library of the endpoint (day 17) were also found to be present using the G2 Phylochip; plus, many additional OTUs were observed. The Phylochip analysis identified a total of 500 OTUs across seven samples (18 phyla, 38 classes, 64 orders, and 91 families) compared to 13 phyla, 18 classes, 17 orders and 32 families detected with the clone libraries. The total fecal community populations of all animals consuming HLZ milk for 14 days were similar to each other, as were the populations of bacteria in the feces of three of the four control-fed animals (Fig. 2). Using principal component analysis (PCA), the six samples were segregated into two distinct clusters. Based on the PCA, one of the control

samples (Con-1 in Fig. 2) was a clear outlier and hence was removed from further statistical analysis. A total of 113 OTUs were significantly different (q [adjusted P value taking into consideration false-discovery rate] ≤ 0.05) across the control and HLZ-fed groups (see Fig. S2 in the supplemental material). Figure 3 summarizes the differences at the family level, which had at least two OTUs that were significantly different between the treatment and control groups (total of 64 significant OTUs). Among all groups, *Clostridiales* were statistically underrepresented in HLZ-fed pigs ($q = 0.069$), while *Bacteroidetes* were overrepresented in HLZ-fed pigs ($q = 0.1$) compared to pigs fed control milk. Interestingly, beneficial microbes such as *Bifidobacteriaceae* (2 OTUs) and *Lactobacillaceae* (2 OTUs) were also enriched upon consumption of HLZ milk whereas detrimental microbes such as *Streptococcaceae* (1 OTU) and *Campylobacterales* (2 OTUs) were depleted with consumption of HLZ milk (see Table S1 in the supplemental material).

DISCUSSION

Human milk contains numerous bioactive components that can modulate the infant gut microbiome and thus benefit the host. We used a model system of pigs and transgenic goat milk containing HLZ to examine the effects of consumption of this antimicrobial milk component on beneficial microbe enrichment versus detrimental microbe reduction in the gut microbial community. We demonstrated that consumption of lysozyme-rich milk indeed altered gut microbiota populations and shifted the microbial population to those microbes associated with activities beneficial for the host. The changes in microbial populations seen can be attributed to the presence of HLZ in milk. Fine analysis of milk composition using 2-D gels indicated that there were no off-target proteins being produced and no endogenous proteins whose production was being diminished by the expression of HLZ and therefore that all effects of the milk were a result of the presence of HLZ. Moreover, human milk oligosaccharides which are known modulators of intestinal microbiota are not found in the same diversity or quantity in goat milk (9), demonstrating that HLZ alone could contribute this important function to the milk of dairy animals.

Microbial abundance increases along the length of the diges-

TABLE 3 Fecal microbial profile after 14 days of consuming HLZ or control milk

Phylum	% of clones in indicated pig group ^a		<i>P</i>
	Control fed (<i>n</i> = 3; 356 clones)	HLZ fed (<i>n</i> = 4; 532 clones)	
<i>Bacteroidetes</i>	46.6	52.8	0.070
<i>Firmicutes</i>	41.6	35.5 ^b	0.038
<i>Proteobacteria</i>	7.3	5.6	0.317
<i>Tenericutes</i>	1.1	0.8	0.537
<i>Spirochaetes</i>	1.1	1.1	1
<i>Actinobacteria</i>	0.3	0	0.321
<i>Lentisphaerae</i>	0	0.4	0.430
<i>Fusobacteria</i>	0	0.6	0.258
Unclassified	2.0	3.2	

^a Data represent the percentages of clones assigned to a phylum using 16S rRNA gene sequencing. Clone values in column headings represent the number of sequences in each library.

^b HLZ-fed pigs significantly different from controls ($P < 0.05$).

TABLE 4 Significant differences in bacterial genera by 16S rRNA gene sequencing after 2 and 14 days of HLZ milk consumption

Phylum	Class	Order	Family	Genus	P	% of total clones in indicated pig group	
						Control fed	HLZ fed
Day 6							
<i>Tenericutes</i>	<i>Mollicutes</i>	<i>Anaeroplasmatales</i>	<i>Anaeroplasmataceae</i>	<i>Anaeroplasma</i>	0.000103	6.1	0.8
<i>Firmicutes</i>	<i>Clostridia</i>	<i>Clostridiales</i>	<i>Ruminococcaceae</i>		0.0114	31.4	38.9
					0.0048	27.4	35.5
					0.105	11.0	14.3
					0.00932	3.8	7.6
					0.174	5.5	7.6
					0.029	0.8	2.7
					0.881	7.2	7.4
<i>Bacteroidetes</i>	<i>Bacteroidia</i>	<i>Bacteroidales</i>		<i>Roseburia</i>	0.00012	3.4	0.3
					0.358	54.0	51.2
				<i>Prevotellaceae</i>	0.0183	50.0	42.8
			<i>Prevotella</i>	0.0008	50.0	35.8	
Day 17							
<i>Firmicutes</i>	<i>Clostridia</i>	<i>Clostridiales</i>	<i>Ruminococcaceae</i>		0.038	42.1	35.5
					0.045	38.5	31.9
					0.317	14.3	12.0
					0.038	1.4	0.2
					0.992	5.6	5.6
					0.012	2.2	0.6
					0.682	1.7	2.1
<i>Bacteroidetes</i>	<i>Bacteroidia</i>	<i>Bacteroidales</i>		<i>Lactobacillaceae</i>	0.671	0.3	0.6
				<i>Streptococcaceae</i>	0.97	1.4	1.5
					0.070	46.6	52.8
				<i>Prevotellaceae</i>	0.035	44.7	52.1
					0.215	39.0	43.2
					0.033	0	1.3
				<i>Porphyromonadaceae</i>	0.097	2.8	5.1
		0.033	0	1.3			

tive tract (35), with the largest diversity and most bacteria found in the colon, and therefore the microbiota of feces is distinct from that of the rest of the GI tract in both pigs (7) and humans (4, 41). Using standard culturing techniques, total coliform and *E. coli*

counts in the duodenum and ileum of HLZ-fed pigs tended to be lower than in control-fed pigs, similar to results seen when starting with 14-day-old pigs (5, 30). These results indicate the reproducibility of the effect of HLZ milk in differently aged animals at this level of detail. As shown using molecular techniques and fecal samples, the overall level of *Proteobacteria* was not significantly lower in HLZ-fed animals than in controls (5.6% versus 7.3%) and there were no differences in *E. coli* levels in the feces of HLZ-fed and control-fed animals, likely a difference in sample location (small intestine versus feces). *Proteobacteria* are a common component of the distal gut, and *in vitro* work has demonstrated that HLZ milk acts in a bactericidal fashion toward *E. coli* (31).

The predominant phyla present in the feces of both feeding groups of pigs at all time points were the *Firmicutes* and *Bacteroidetes*, similar to previous studies with both pig and human subjects (7, 13, 23), suggesting that the pig is a good animal model for studying the GI microbiome and the consequences of microbiota manipulation. The microbial populations were not statistically different between individuals at the start of the trial (day 1, no milk) at the phylum level, indicating that all pigs were starting with similar fecal microbiotas. It should be noted that while samples were prepared after storage at 4°C, which has been shown to have an impact on detected microbial diversity (36), all samples were subjected to similar storage conditions, thus allowing direct comparison between samples. After the 3-day period of adjust-

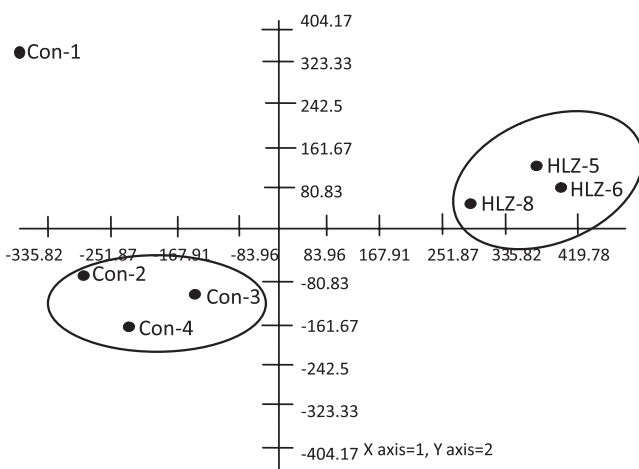


FIG 2 Projection of samples on the first two principal component axes based on the PCA of OTU abundance from the Phylochip analysis. HLZ, pigs fed milk from HLZ-transgenic animals; Con, pigs fed milk from nontransgenic control animals.

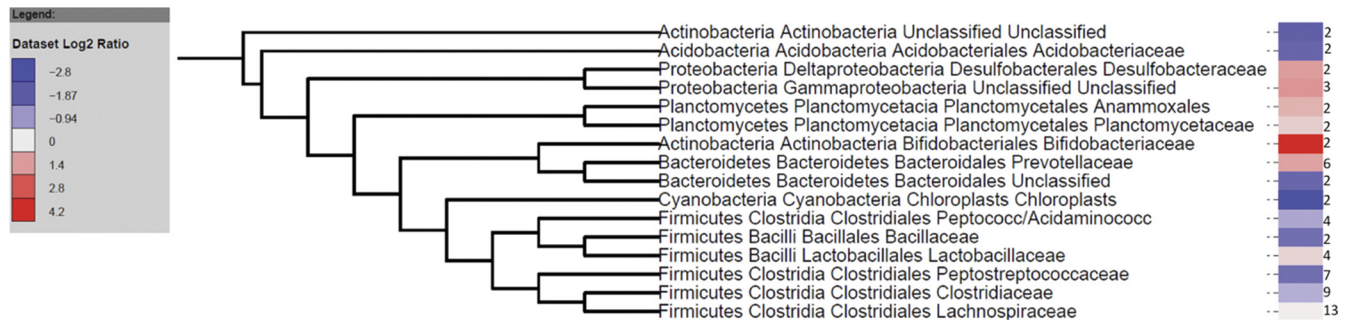


FIG 3 Summarized \log_2 ratios for families that had at least two significantly different ($q \leq 0.05$) OTUs between the control and HLZ-fed treatment groups at day 17 as determined by Phylochip analysis. The numbers beside the heat map represent the OTUs that were significantly different in each family.

ment to consumption of milk, there were significantly more *Proteobacteria* (namely, *Succinivibrio*) present than on day 1, but there were no significant differences between animals. While milk alone altered the fecal microbiota, all animals started with similar microbiotas before the administration of HLZ milk and thus all subsequent changes seen were due to direct effects of HLZ milk and not natural variation. The elevated level of *Succinivibrio* seen after consumption of milk commenced was maintained over all later time points in both feeding groups. *Succinivibrio* commonly inhabit the rumen of dairy animals to aid in digestion, and thus, consumption of goat milk in general increased levels of this microbe either by stimulating the growth of the low levels of *Succinivibrio* already present or by reducing the levels of other microbes, thereby allowing more growth of *Succinivibrio*. Using 16S rRNA gene sequencing of clone libraries, no *Succinivibrio* bacteria were found to be present in the milk itself (data not shown).

Previous work has demonstrated that a majority (95%) of the *Firmicutes* in the human gut were *Clostridia* (13). Similar findings were seen here, as after 14 days of milk consumption, 91% of the *Firmicutes* in control-fed animals were *Clostridia*. In HLZ-fed animals, levels of *Clostridia* were reduced from 92.4% (day 2) to 89.5% of total *Firmicutes* at day 17. While *Firmicutes* are important for supplying energy in the form of short-chain fatty acids, a skewed ratio of *Firmicutes* to *Bacteroidetes* has been implicated in obese mice and humans (25, 26), with elevated levels of *Firmicutes* and decreased levels of *Bacteroidetes* in obese subjects. An increase in *Firmicutes* has been associated with an increased ability to extract energy from the diet and/or promote the deposition of this energy in the form of fat (19, 45). After 14 days of HLZ milk consumption, the proportion of *Firmicutes* in the fecal microbiota was underrepresented and that of *Bacteroidetes* overrepresented compared to animals consuming control goat milk. The gut surface area of the animals used in this study was investigated previously and was found to be related to an improved absorptive capacity of the intestinal epithelium, as HLZ-fed pigs tended to have longer villi and had a significantly thinner lamina propria in the duodenum (10). A 20% increase in the proportional representation of *Bacteroidetes* in fecal microbiota in humans has been correlated with a decrease in nutrient absorption (19). Here we observed a more modest increase in fecal *Bacteroidetes* content (6.2%) upon consumption of HLZ milk that was related to changes in cellular ultrastructure in the small intestine. While the energy content of the feces was not determined in this study, the resulting histological changes represent an intestinal epithelium better able to absorb what nutrients were present if less energy was

indeed available due to the underrepresentation of the energy supplying *Firmicutes*. Combined, these results suggest that microbiota composition can influence the physical state of the intestine. It remains to be determined what other metabolic and functional changes occur in response to HLZ-induced modulation of gut microbiota.

One important issue regarding gut microbiota communities is their stability over time (3). Animals fed control milk were more prone to fluctuation in the relative proportions of phyla present after the 3-day adjustment period. The significant increase in *Tenericutes* at day 6 in control-fed animals was related to one individual; however, the proportions of rest of the phyla were more consistent between individuals of the same feeding group. Gut microbiota populations have been reported to have minimal variation over time within individuals but are highly variable between people (11). The greater fecal community member stability seen with HLZ milk consumption may have been due to the presence of HLZ acting to quiet challenges to the gut, the short interval between sample analysis, or a brief environmental perturbation, although all animals were housed together and exposed to the same environment. Changes in diet have been shown to rapidly induce changes in fecal microbiota (within 3 to 4 days), after which the microbiota was maintained for several weeks until the diet was altered again (48). Here, the introduction of milk altered fecal microbiota in a similarly short time frame; however, the changes were not static. The changes being seen in both feeding groups over time may reflect natural variation in microbiota stability over a shorter time period and point to HLZ being able to continuously modulate community member diversity in a similar pattern over time.

Overall, the G2 Phylochip analysis supported our findings obtained with 16S rRNA gene sequencing and offered more details. Both methods identified *Bacteroidetes* and *Firmicutes* as the main phyla and the presence of fewer *Firmicutes* and more *Bacteroidetes* in HLZ-fed animals compared to control-fed animals. Within phyla and families, levels of community members were both increasing and decreasing in response to HLZ milk consumption, with the exception of *Bacteroidetes*, where levels of all members were elevated compared to the results from control-fed animals. Greater variability within phyla has been reported in previous studies (13); however, HLZ milk was also able to change the bacterial abundance at the phylum level.

Lysozyme-rich milk was capable of significantly altering community member diversity in a fashion that allowed identification of the feeding group based on the resulting fecal microbiota.

Whether these changes can influence overall health and resistance to disease remains to be determined, but the types of changes seen are associated with improved gut health. The more comprehensive data generated with the Phylochip demonstrated that, compared to the microbiota of control-fed pigs, the microbiota of pigs fed HLZ-rich milk more closely resembled that of human infants being breast fed, with the enrichment of *Bifidobacteriaceae* and *Lactobacillaceae*, both biomarkers of increased gut and host health (47). These beneficial changes were accompanied by the reduction of the numbers clostridia and *Streptococcaceae*, which are components of the fecal microbiota of infants fed formula that lacks lysozyme, as well as by decreased levels of disease-related bacteria such as *Mycobacteriaceae* and *Campylobacterales*. It is likely that the antimicrobial action and/or cationic properties of HLZ were contributing to the selection of these beneficial bacteria by preventing the growth of others. Taken as a whole, the data suggest that addition of HLZ alone to milk is able to modulate intestinal microbiota in a fashion that is similar to the modulation seen with human milk.

Untangling the complex interactions of nutrient, host, and bacteria is a substantial challenge. The results of this study support the hypothesis that milk components actively modified the gut microbiome, resulting in community membership changes to increase levels of beneficial microbes and reduce levels of undesirable community members. They also support the use of pigs as a model animal for gut microbiota research and, along with the use of HLZ-rich milk, offer an approach not only for the study of the mechanism of action of lysozyme at the level of the gut but also for the direct manipulation of gut microbiota with the potential to improve GI disorders, including diarrhea and inflammatory bowel diseases such as Crohn's disease and colitis.

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