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Consumption of pasteurized human lysozyme transgenic goats' milk alters serum metabolite profile in young pigs

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Abstract Nutrition, bacterial composition of the gastrointestinal tract, and general health status can all influence the metabolic profile of an organism. We previously demonstrated that feeding pasteurized transgenic goats' milk expressing human lysozyme (hLZ) can positively impact intestinal morphology and modulate intestinal microbiota composition in young pigs. The objective of this study was to further examine the effect of consuming hLZ-containing milk on young pigs by profiling serum metabolites. Pigs were placed into two groups and fed a diet of solid food and either control (non-transgenic) goats' milk or milk from hLZ-transgenic goats for 6 weeks. Serum samples were collected at the end of the feeding period and global metabolite profiling was performed. For a total of 225 metabolites (160 known, 65 unknown) semi-quantitative data was obtained. Levels of 18 known and 4 unknown metabolites differed significantly between the two groups with the direction of change in 13 of the 18 known metabolites being almost entirely congruent with improved health status, particularly in terms of the gastrointestinal tract health and immune response, with the effects of the other five being neutral or unknown. These results further support our hypothesis that consumption of hLZ-containing milk is beneficial to health.

Keywords Lysozyme · Milk · Genetic engineering · Metabolite profiling

Introduction

A myriad of cellular processes are required for the normal growth, maintenance, and function of a cell or organism. Metabolites, small, low molecular weight compounds, are integral to these processes and can be of endogenous origin, or may be xenobiotic in nature, coming from food or pharmaceuticals (Dunn 2008; Gibney et al. 2005). The field of metabolomics involves the global profiling, identification, quantification, and study of all metabolites, or as many as possible, in a specific tissue or matrix of an organism under a defined set of conditions at a certain point in time (Whitfield et al. 2004; Goodacre 2007).

The metabolic profile of an organism can serve as an indicator of its physiological state at a given time point (Whitfield et al. 2004). The presence of specific metabolites and their levels is dependent upon the

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inputs and interactions between a number of internal and external factors; therefore, changes in any of these factors are reflected in the composition of the metabolome (Gibney et al. 2005; Whitfield et al. 2004; Goodacre 2007). Diet and nutrition, in particular, can both have major effects on the metabolome (Gibney et al. 2005). Various studies have documented that changes in consumption of certain foods, including milk, may result in subsequent changes in metabolic profiles (Roura et al. 2008; Bertram et al. 2007). Another major contribution to the metabolome of humans and other mammals is the gastrointestinal tract (GI) microbiota (Gibney et al. 2005; Goodacre 2007), which may contain an estimated 10^{13}–10^{14} organisms from more than 1,000 species (Gill et al. 2006). These microbiota have their own metabolic networks and processes (Gill et al. 2006; Nicholson et al. 2004), and as such, can supply metabolite products directly to their host (Gibney et al. 2005; Goodacre 2007; Gill et al. 2006). The microbes can also metabolize molecules already processed by the hosts, or other microbes, transforming them and creating new metabolites. The metabolic functions of the microbial population are necessary for maintaining the host's health as they are capable of synthesizing nutrients essential to their mammalian hosts (Gibney et al. 2005; Gill et al. 2006). Microbial metabolites from intestinal microbiota have been detected in both human serum and urine (Nicholson et al. 2004), while Li et al. (2008) have shown that differences in GI microbial populations are reflected in changed urine metabolic profiles from human research subjects.

Recent studies have demonstrated a link between the GI tract microbial population, metabolic profiles, and the host's health. Feeding *Lactobacillus* species to rats and mice affected lipid metabolite levels in the blood and liver and overall had an anti-diabetic effect (Yadav et al. 2007). Differences in the microbiota and the effect on the metabolome are also correlated with obesity and cardiovascular disease (Ley et al. 2006; Ordovas and Mooser 2006). In addition, Martin et al. (2007) showed that introducing a human baby flora to mice resulted in changes in the metabolomes of several different tissues, including plasma, liver, and the gut.

Lysozyme, a 1,4-β-N-acetylmuramidase, cleaves a glycosidic linkage in the peptidoglycan component of bacterial cell walls, ultimately causing the cell membrane to lose integrity and the cell to die (Ellison and Giehl 1991; Blake et al. 1967). Notably, lysozyme is an important non-specific immune defense factor in human milk, where it occurs at high levels (400 mg l^{-1}) compared to the milk of dairy animals (Chandan et al. 1968). Studies indicate that lysozyme contributes to the establishment of the bifidobacteria- and lactobacilli-rich GI biota in breastfed infants that is protective against GI illness and may aid in the maturation of the intestinal tract (Coppa et al. 2006; Solis et al. 2002; Schiffrin and Blum 2002; Newburg and Walker 2007). Lysozyme is active against a number of bacteria, particularly gram-positive species; however, it has also been shown to be effective against gram-negative species in vitro (Ellison and Giehl 1991; Masschalck and Michiels 2003; Maga et al. 2006a). Evidence suggests that lysozyme may be involved in modulating the inflammatory response as well (Brandenburg et al. 1998; Ginsburg 2002; Takada et al. 1994a, b; Muller et al. 2005). Also, addition of lysozyme to the diet has been shown to increase feed efficiency and positively affect the GI morphology in young chicks (Humphrey et al. 2002).

Bovine and caprine milk have very low levels of lysozyme (0.16 and 0.23 mg l^{-1}, respectively) relative to human milk (Chandan et al. 1968). Our lab previously developed a line of transgenic dairy goats which express hLZ at 67% (270 mg l^{-1}) of the level found in human milk (Maga et al. 2006a). We have shown that feeding pasteurized milk from these goats can positively impact the health of young pigs and goats (Maga et al. 2006b; Brundige et al. 2008). In both species, consumption of hLZ-containing milk significantly changed the population of *E. coli* and coliforms in the small intestine (Maga et al. 2006b). In a second study, several measures of GI health, including morphology and duodenum villi intraepithelial lymphocyte count, were improved in weaned young pigs that were reared on a diet that included hLZ-containing milk (Brundige et al. 2008). Considering that changes in diet, GI microbiota, and general health status are reflected in changes in the metabolome, and taking into account the previous outcome we saw in the young pigs that consumed hLZ transgenic milk, we profiled serum metabolites in order to further investigate the effects of pasteurized hLZ transgenic milk on young pigs.

Materials and methods

Animals and diet

We previously described the pigs used for this metabolomic profiling study, their selection, diet, and treatment during rearing (Brundige et al. 2008). Briefly, at 12 days of age, 12 pigs were weaned from sows and distributed into two feeding groups, each balanced for weight, sex, and genetic background. Two pools of milk, one from hLZ transgenic does and one from non-transgenic control does, were formed prior to pasteurization. All milk was pasteurized prior to feeding to the pigs. The pigs were fed 8 oz (0.24 l) of goat milk per animal twice a day, and solid feed and water were available ad libitum to all animals throughout the study. The amount of solid food consumed by pigs was not measured. Pigs were first weaned onto Pig A2000 Pellet Denagard/CTC starter diet (Akey, Lewisburg, OH, USA), containing lactose, cereal food fines, soybean meal, oat groats, ground corn, animal plasma, poultry meal, fish meal, cheese meal, vegetable and animal fat, and the antibiotics tiamulin hydrogen fumarate and chlorotetracycline. This diet provided 21% crude protein, 8% crude fat, and 2% crude fiber. After 2 weeks, the pigs were switched to a standard grower diet (Associated Feed, Turlock, CA, USA). The grower diet contained wheat millrun, fat mixer, ground corn, blood meal, whole dried whey, soybean meal, Akey Swine Micro 4 mix, and Tylan 40 antibiotic. This diet provided 20% protein, 7% crude fat, 2% crude fiber, and ME^2 of 13.6 MJ kg^{-1}. All animals under study were monitored at each feeding for any signs of illness and were cared for and housed under AAALAC-approved conditions. After 6-weeks, the pigs were necropsied at the California Animal Health and Food Safety Laboratory (UC Davis, Davis, CA, USA) by an American College of Veterinary Pathologist (ACVP) board-certified animal pathologist.

Blood collection and serum preparation

Whole blood was collected from each animal immediately before necropsy via venipuncture into additive-free blood collection tubes (Vacutainer, BD). Samples were allowed to clot for 4 h at 4°C. After coagulation, samples were centrifuged in 1.5 ml aliquots at 1,500 RPM to separate serum from clotted material. The serum aliquots from each pig were pooled post-centrifugation and before transferring to clean microcentrifuge tubes for storage at −70°C until use.

Metabolite profiling

Three types of mass spectrometry analysis were applied to all samples: GC-MS (gas chromatography-mass spectrometry), LC-MS/MS (liquid chromatography-mass spectrometry/mass spectrometry), and SPE-LC-MS/MS (Solid phase extraction-LC-MS/MS). For mass spectrometry-based metabolite profiling analyses proteins were removed from plasma samples by precipitation. Subsequently samples were extracted and polar and non-polar fractions were obtained by adding water and a mixture of ethanol and dichloromethane. For GC-MS analysis, the non-polar fraction was treated with methanol under acidic conditions to yield fatty acid methyl esters. Both fractions were further derivatized with O-methyl-hydroxyamine hydrochloride and pyridine to convert oxo-groups to O-methyloximes and then subsequently with a silylating agent before analysis. For LC-MS analysis, both fractions were reconstituted in appropriate solvent. High performance liquid chromatography (HPLC) was performed by gradient elution using methanol/water/formic acid on reversed phase separation columns. Mass spectrometric detection technology which allows targeted and high sensitivity multiple reaction monitoring (MRM) profiling was performed in parallel to a full screen analysis. Steroids and their metabolites were measured by online SPE-LC-MS/MS. Catecholamines and their metabolites were measured by online SPE-LC-MS/MS as described by Yamada et al. (2002). For both, absolute quantification was performed by means of stable isotope-labeled standards.

Data analysis and statistics

Reference samples (pool samples) were generated from equal amounts of individual experimental samples and ran through the pre-analytical and analytical process together with individual samples. GC-MS and LC-MS/MS profiling data were expressed as

ratios relative to pool samples. For catecholamines and steroids, quantitative data (levels in ng ml^{-1}) were obtained, except for cortisol, which was normalized to pool samples.

Metabolite profiling data was analyzed by using univariate statistics to determine differences between treatment groups. All data were log10-transformed. Means of the treated groups were compared to the means of the respective untreated control groups using Student's t-test ($P < 0.05$). Median values were calculated for each treatment group as were a ratio of median values as a measure of effect size and direction. t-tests were calculated on the basis of log-transformed data to ensure normal distribution. Heteroskedastic t-test (Welch test, assuming unequal variance between treatment groups) were calculated, and a significance level of $P < 0.05$ was considered significant. The P value was not adjusted for multiple testing; therefore, results must be viewed in comparison to the false-positive rate, i.e. at $P = 0.05$ and 225 metabolites, 11.25 significant changes would be expected to arise by chance, whereas 22 were observed.

Results

We quantified a total of 225 metabolites in the pig serum samples analyzed for this study. We have identified 160 of these metabolites, with the remaining 65 metabolites being unknown. The 160 known metabolites fell into 16 classes: amino acids; amino acid derivatives; carbohydrates and related; energy metabolism and related; nucleobases and related; cholesterol, bile acids, fatty alcohols, and related; fatty acids, glycerides (di-, and triglycerides); glycolipids; phospholipids; sphingolipids; catecholamines and other monoamines; steroids and related; other hormones, signal substances and related; vitamins, cofactors, and related; and miscellaneous metabolites. None of the metabolites identified were significantly different between the pigs fed hLZ-milk and those fed control milk in seven metabolite classes (nucleobases and related; cholesterol, bile acids, fatty alcohols, and related; glycerides; glycolipids; sphingolipids; other hormones, signal substances, and related; and steroids). Levels of 18 known metabolites and 4 unknown metabolites were significantly different in pigs given hLZ milk

compared to pigs that received control milk (Table 1). The levels of 17 metabolites were significantly increased, while the levels of five metabolites were decreased.

Discussion

Differences in diet, nutrition, and health can be reflected in the profile of serum metabolites in an organism. We have previously shown that pasteurized hLZ-containing milk from transgenic goats can positively affect the health of young pigs by changing the bacterial composition and improving the morphology of the GI tract (Brundige et al. 2008; Maga et al. 2006b). The results of this metabolic profiling experiment further support the hypothesis that consumption of hLZ-containing milk imparts positive health benefits to the young animal.

Amino acids

We found that methionine and threonine, both essential amino acids, were significantly increased in hLZ-milk fed pigs as compared to controls. Methionine and its metabolite products play critical roles in a number of cellular functions including immune response, DNA/RNA synthesis and repair, and a number of other biochemical reactions necessary for normal cellular function (Grimble and Grimble 1998; Grimble 2006; Lu and Mato 2008). Threonine, like methionine, is important to immune defense as high levels are needed to support protein synthesis during the acute phase of immune response (Faure et al. 2007). Threonine is also important to gut health as increased levels correlate to increased synthesis of mucosal proteins important for maintaining intestinal mass and integrity (Nichols and Bertolo 2008). Increased demand for methionine and threonine during illness results in a decrease in plasma concentrations. The increased levels that we observed are consistent with a less activated immune system, in which fewer immune responses are necessary to fight invading pathogens.

Amino acid derivatives

Four amino acid derivatives significantly differed between the two groups of pigs. The levels of 3-

Table 1 Significant known metabolite changes observed between hLZ-fed and control-fed pigs

Metabolite	hLZ mean ± SD	Control mean ± SD	hLZ median	Control median	Median ratio (hLZ/control)	Median ratio P value
Amino acids						
Methionine	0.991 ± 0.102	0.605 ± 0.083	0.885	0.543	1.464	0.003
Threonine	0.942 ± 0.089	0.730 ± 0.062	0.877	0.712	1.201	0.039
Amino acid derivatives						
3-Hydroxyindole[a]	0.336 ± 0.097	0.625 ± 0.108	0.367	0.618	0.587	0.001
Urea	0.689 ± 0.062	0.999 ± 0.093	0.685	0.919	0.686	0.008
trans-4-hydroxyproline	1.078 ± 0.065	0.826 ± 0.066	1.112	0.829	1.345	0.012
Citrulline	1.359 ± 0.106	0.962 ± 0.132	1.386	0.950	1.440	0.049
Carbohydrates and related						
1,5-Anhydrosorbitol	1.249 ± 0.051	0.833 ± 0.083	1.287	0.820	1.545	0.002
Scyllo-inositol	0.820 ± 0.102	0.602 ± 0.082	0.801	0.575	1.330	0.035
Myo-inositol	0.675 ± 0.124	0.467 ± 0.103	0.650	0.471	1.393	0.041
Energy metabolism and related						
Alpha-ketoglutarate	1.249 ± 0.132	0.795 ± 0.116	1.187	0.799	1.492	0.024
Fatty acids						
Arachidonic acid	1.116 ± 0.038	0.919 ± 0.070	1.135	0.929	1.236	0.027
Phospholipids						
Phosphatidylcholine #2[b]	1.059 ± 0.046	0.930 ± 0.027	1.035	0.915	1.113	0.035
Catecholamines						
3-Methoxytyrosine	37.400 ± 0.020	40.567 ± 0.029	37.500	40.500	0.924	0.038
Vitamins, co-factors, and related						
Coenzyme Q10	1.148 ± 0.053	0.700 ± 0.164	1.132	0.639	1.618	0.014
Miscellaneous						
Taurine	1.210 ± 0.163	0.651 ± 0.088	1.135	0.624	1.745	0.011
Benzoic acid	0.936 ± 0.021	1.031 ± 0.027	0.946	1.011	0.918	0.014
2-Hydroxybutyrate	1.044 ± 0.088	0.769 ± 0.036	1.066	0.752	1.386	0.015
Creatinine	0.874 ± 0.042	1.035 ± 0.052	0.862	0.988	0.832	0.024

[a] Measurement may include a possible contribution from 3-indoxylsulfuric acid

[b] Structure annotation is based on strong analytical evidence (combinations of chromatography, mass spectrometry, chemical reactions, deuterium-labeling, database and literature search, as well as comparisons to similar/homologue/isomeric reference compounds)

hydroxyindole (minor: 3-indoxylsulfuric acid) and urea were significantly lower in pigs fed hLZ transgenic milk while trans-4-hydroxyproline and citrulline were increased. If inadequate levels of amino acids are present in the diet, the body catabolizes proteins, which leads to an increase in urea production (Singh 2007). Multiple studies in pigs have shown that decreased muscle catabolism correlates to a decrease in serum and plasma levels of urea (Vann et al. 2000; Davis et al. 2004; Bush et al. 2002; Dunshea et al. 1992). The decrease in urea in the hLZ-milk fed pigs is indicative of decreased

muscle catabolism and increased utilization of amino acids.

3-Hydroxyindole is an intermediate in the conversion of tryptophan to indoxyl sulfate by gut microbiota (Sims and Renwick 1985; Gillam et al. 2000). Indoxyl sulfate is a toxic compound that can contribute to renal failure (Banoglu and King 2002) and cause oxidative stress in endothelial cells (Dou et al. 2007). Although indoxyl sulfate was lower, it was not significantly so (hLZ/control median ratio, 0.797, $P = 0.301$). The lower level of 3-hydroxyindole is likely a result of a changed microbiota in the
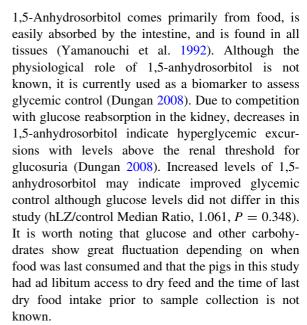
GI tract. While the decrease of a precursor for a toxic compound is desirable in terms of health, the full extent of changes in gut microbiota populations is unknown at this point.

Hydroxyproline is a major component of collagen, where it helps to maintain stability (Krane 2008). Although it is found in few other mammalian proteins, hydroxyproline is notably present in elastin and the C1q fraction of complement (Haskell and Johnston 1991). Because hydroxyproline is formed from a post-translational modification, free hydroxyproline in biofluids comes primarily from the breakdown of collagen and elastin, although C1q may also contribute a significant amount (Garnero and Delmas 1996). Collagen break-down products in serum are a marker of bone resorption, but they can also come from collagen degradation due to inflammation (Weinberger et al. 2005). Bone remodeling and turnover is high during stages of rapid growth (Szulc et al. 2000), such as occurs in young pigs; however increased growth as measured by body weight was not observed in the hLZ-milk fed pigs (Brundige et al. 2008). Similarly, we saw no evidence of a systemic immune response in hLZ-milk fed pigs as measured by changes in white blood cell counts (Brundige et al. 2008), nor did we see changes in the levels of mRNA of key tissue regulators of inflammation (unpublished data). Thus, the significance of increased levels of trans-4-hydroxyproline in the hLZ-milk fed pigs is unclear, although it likely reflects the remodeling of bone and other connective tissue during growth.

Citrulline is produced both during the urea cycle and from the oxidation of arginine by nitric oxide synthases (Muriel 2000). Citrulline is a marker of intestinal absorption and function, with increased levels correlating with increased absorptive function (Jianfeng et al. 2005). Increased serum citrulline levels are also correlated to morphology changes and increases in absorptive surface area of the intestine (Santarpia et al. 2008; Crenn et al. 2008), such as seen in the hLZ-milk fed pigs (Brundige et al. 2008). Thus, the increased level of citrulline observed in the serum of the hLZ-milk fed pigs is consistent with improved GI health.

Carbohydrates and related

1,5-Anhydrosorbitol, scyllo-inositol, and myo-inositol were higher in hLZ-fed animals than in controls.

1,5-Anhydrosorbitol comes primarily from food, is easily absorbed by the intestine, and is found in all tissues (Yamanouchi et al. 1992). Although the physiological role of 1,5-anhydrosorbitol is not known, it is currently used as a biomarker to assess glycemic control (Dungan 2008). Due to competition with glucose reabsorption in the kidney, decreases in 1,5-anhydrosorbitol indicate hyperglycemic excursions with levels above the renal threshold for glucosuria (Dungan 2008). Increased levels of 1,5-anhydrosorbitol may indicate improved glycemic control although glucose levels did not differ in this study (hLZ/control Median Ratio, 1.061, $P = 0.348$). It is worth noting that glucose and other carbohydrates show great fluctuation depending on when food was last consumed and that the pigs in this study had ad libitum access to dry feed and the time of last dry food intake prior to sample collection is not known.

Myo-inositol is present in a wide variety of foods, including grains, fruits, vegetables, and nuts (Clements and Darnell 1980). Scyllo-inositol is an isomer of myo-inositol that is found free in small amounts and incorporated into phytic acid in plants (Anderson and Wolter 1966). Myo-inositol is readily absorbed by the intestine (Clements and Reynertson 1977), and increasing its dietary availability positively correlates to increased serum levels (Clements and Darnell 1980). Although myo-inositol can be synthesized in vivo from glucose-6-phosphate (Holub 1982), a portion of inositol comes from phytate degradation by certain species of intestinal bacteria (Steer et al. 2004; Rodriguez et al. 1999). The increases seen in the hLZ transgenic milk-fed pigs could possibly be due to changed bacterial composition in the GI tract, with an increase in species possessing phytase ability. Although the change was not significant, phosphate levels in the pigs who consumed hLZ-containing milk showed a slight, near-significant increase (hLZ/control median ratio, 1.117, $P = 0.059$). The increased serum levels seen for both scyllo- and myo-inositol may have some health benefits. For example, scyllo-inositol has been shown to reverse memory deficit in transgenic mice with an Alzheimer's phenotype (Fenili et al. 2007). Myo-inositol has a number of physiological effects including preventing fatty liver, reducing cholesterol, and improving calcium absorption (Pallauf and Rimbach 1997). Myo-inositol metabolites also function as critical secondary

messengers in cell signaling (Downes and Macphee 1990).

Energy metabolism and related

Alpha-ketoglutarate, an energy metabolite, was increased in hLZ-fed animals. Alpha-ketoglutarate is formed during the Krebs cycle and can also be synthesized by certain anaerobic bacteria, including several species of *Bacteriodes* (Allison et al. 1979). Increased levels have been associated with a number of benefits including: increased bone growth as measured by the length of the 6th rib in pigs (Andersen et al. 2008), decreased cholesterol levels in rats (Radzki et al. 2009), protection of gut mucosa and kidney function (Dabek et al. 2005), and possible prevention of inappropriate muscle catabolism during times of stress, such as surgery, injury and illness (Wiren et al. 2002). As with several other observed metabolite differences, the increase in alpha ketoglutarate is likely due to changes in the composition of the intestinal microbe population.

Fatty acids

Arachidonic acid (ARA) was the only fatty acid measured which was significantly different, being elevated in the hLZ-milk fed pigs. ARA is a precursor of inflammatory mediators and is involved in the function of several organs and systems either directly or via conversion to eicosanoids (Khanapure et al. 2007; Pompeia et al. 2003). Increased levels of ARA metabolites are usually considered harmful as they can lead to enhanced aggregation of platelets, endo-peroxides, and thromboxanes (Khanapure et al. 2007). As previously mentioned, qRT-PCR data did not show increases in key pro-inflammatory cyto-kines in intestinal tissue (unpublished data), nor did complete blood count data or pathology results indicate an inflammatory response (Brundige et al. 2008). ARA is also critically important for proper brain and neural development during infancy and early childhood, particularly in the first 2 years when brain growth is most rapid (Kurlak and Stephenson 1999; Martinez 1992). Although neonates can synthesize ARA, it is needed at such high levels that it is considered a dietary essential fatty acid (Salem et al. 1996; Wainwright 2002). Because lipid metabolism in young pigs is similar (Innis 1993) and brain growth

patterns and timing are similar between young pigs and humans, pigs are often considered a good model species for use in assessing links between nutrition and brain development (Innis 1993; Dobbing and Sands 1979). Huang et al. (2007) have shown that increasing ARA in the diet of young pigs causes plasma levels to also rise. Given the histological and metabolic indicators of improved absorptive capacity of the GI tract, the higher level of ARA observed in the hLZ-fed pigs could potentially be due to increased absorption in the intestine.

The serum levels of phosphatidylcholine #2 and the DOPA catabolite 3-methoxytyrosine were also significantly altered, but the possible significance of these changes are not known.

Vitamins, co-factors, and related

Serum levels of co-enzyme Q10 were significantly increased in hLZ-fed animals. Co-enzyme Q10 has multiple critical roles in normal cellular function and maintenance (Littarru and Tiano 2007). It serves as an electron carrier in the mitochondrial respiratory chain; is one of the most important lipophilic antioxidants, preventing oxidative damage to cells and DNA; helps to prevent atherosclerosis and other cardiovascular problems; and has neuroprotective properties (Littarru and Tiano 2007; Young et al. 2007). Studies also indicate that co-enzyme Q10 is required for proper function and maintenance of the immune system. Serum levels of co-enzyme Q10 decrease in many pathologic conditions, such as cancer, AIDS, and asthma (Folkers et al. 1997, 1988; Gazdik et al. 2002). Additionally, co-enzyme Q10 is necessary to support appropriate levels of circulating T4 lymphocytes and IgG antibodies (Folkers et al. 1993), both of which play important roles in immune defense (Majlessi et al. 2008; Goldman et al. 1985). Because of its large size and hydrophobic properties, absorption of co-enzyme Q10 is somewhat limited in the intestine (Zhang et al. 1995). The increase seen here may be due to greater intestinal absorption by the pigs fed hLZ-containing milk or to altered co-enzyme Q10 synthesis.

Miscellaneous metabolites

Taurine and 2-hydroxybutyrate were increased in hLZ-milk fed pigs, while benzoic acid and creatinine

were decreased. Taurine is considered essential in pre-term and newborn infants (Verner et al. 2007), and plays a necessary role in many cellular and physiological processes (Bouckenooghe et al. 2006). Taurine conjugates with bile acids to form bile salts needed for the absorption of fatty acids and fat soluble vitamins (Verner et al. 2007). Decreased immune system activity is associated with increased levels of circulating taurine (Stapleton et al. 1998), further supporting the interpretation of a less activated immune system in the hLZ-milk fed animals.

Benzoic acid is produced as a by-product of phenylalanine metabolism in bacteria or when gut bacteria process polyphenols ingested from fruits and beverages. It is commonly used as a food preservative and is generally recognized as safe. Current evidence is unclear if benzoic acid has any affect on health, either positive or negative (Nair 2001). 2-Hydroxy-butyrate (2-hydroxybutryic acid) is synthesized by GI tract bacteria, *Fusobacterium vivarium* (Potrykus et al. 2008). It is also unclear what affect this increase might have on health. The decrease in benzoic acid and increase in 2-hydroxybutyrate are both likely indicators of changed bacterial composition of the GI tract in the hLZ-milk fed pigs.

Creatinine is produced when creatine phosphate is broken down in muscle (Wyss and Kaddurah-Daouk 2000). Serum creatinine levels are positively correlated with muscle mass (Wyss and Kaddurah-Daouk 2000), but elevated levels can also be indicative of impaired renal function (Rule 2007). It is unclear what the relatively lower level of creatinine in the hLZ-fed pigs may indicate.

Summary

We found 18 known and 4 unknown metabolites whose levels in serum were significantly affected by the consumption of hLZ-containing milk from transgenic goats. Of the 18 known metabolites, 14 were changed in a direction consistent with improved health of the animal, although one, ARA, could also possibly be interpreted as a negative (Table 2). The affects of the remaining four (phosphatidylcholine #2, 3-methoxytyrosine, 2-hydroxybutyrate, and benzoic acid) are unclear.

The serum levels of six metabolites (2-hydroxy-butyrate, 3-hydroxyindole, myo-inositol, scyllo-inositol, alpha-ketoglutarate, and benzoic acid) most likely reflect changes in the bacterial composition of

Table 2 Summary of area of impact, direction of change (increased ↑ or decreased ↓), and probable direction of impact (positive + or negative −) for significantly altered metabolites

Area of impact	Metabolite	Direction of change	Direction of impact
GI microbial composition	Benzoic acid	↓	Neutral
	2-Hydroxybutyrate	↑	Neutral
	3-Hydroxyindole	↓	+
	Myo and scyllo-inositol	↑	+
Healthier GI tract	Threonine	↑	+
	Citrulline	↑	+
	Taurine	↑	+
	α-Ketoglutarate	↑	+
Increased growth	Methionine	↑	+
	Threonine	↑	+
	Urea	↓	+
	Creatinine	↓	+/−
	Hydroxyproline	↑	+
	α-Ketoglutarate	↑	+
Immune system	Methionine	↑	+
	Threonine	↑	+
	Taurine	↑	+
	Coenzyme Q10	↑	+
	Arachidonic acid	↑	+/−

the GI tract as a result of hLZ milk consumption and, therefore, the composition of metabolites available for absorption. While it is not clear if there are any consequences from the increased level of 2-hydroxybutyrate or decreased level of benzoic acid, there are systemic benefits to increased levels of scyllo-inositol, myo-inositol, and alpha-ketoglutarate, while decreased levels of 3-hydroxyindol may have a benefit in reduced production of the cellular toxin indoxyl sulfate.

The direction of change of four metabolites (methionine, threonine, hydroxyproline, and urea) suggests increased growth in the pigs reared on hLZ milk. Decreased levels of urea indicate less muscle catabolism, while increased methionine and threonine reflect a greater availability for protein synthesis. The increase in hydroxyproline may suggest enhanced skeletal development.

Changes in four metabolites (threonine, citrulline, taurine, and alpha-ketoglutarate) support improved GI health. Taurine and alpha-ketoglutarate are helpful in protecting the gut mucosal surface, while threonine is important for maintaining intestinal mass and integrity. Increased levels of citrulline are a marker of increased intestinal absorption, which correlates to our previous finding of increased villi width and presumptively surface area in the duodenum (Brundige et al. 2008). Additionally, increases in ARA and coenzyme Q10 may be due to the suggested increase in absorptive capacity of the intestine.

Significant differences in levels of five metabolites (methionine, threonine, taurine, coenzyme Q10, and ARA) are associated with the immune system. The increase in ARA could be a negative as it is associated with increased inflammation and eicosanoids; however, CBC, qRT-PCR data, and pathology results do not support this. However, increases in methionine, threonine, and taurine suggests that the immune systems of the pigs fed hLZ-containing milk were less active than those of the pigs reared on control milk. Also, the increase in coenzyme Q10 may be beneficial in supporting adequate levels of important T4 lymphocytes and IgG antibodies.

In summary, the observed changes in serum levels of 15 of the 18 known metabolites support our previous findings and our hypothesis that the consumption of hLZ-containing milk from transgenic goats provides positive health benefits. While these results are encouraging, the observations to date clearly suggest that the metabolic changes in animals consuming hLZ-containing milk need to be more fully characterized. We have proposed that changes in intestinal microbe population, increased absorption of nutrients from the diet by the intestine, or less immune system activity are driving the differences in metabolite profiles. Additional studies are needed to determine if these speculative causes are indeed behind the changed metabolites or if other mechanisms are responsible. Also, changes in the metabolite profile need to be determined under various conditions, particularly during acute challenge with enteropathogenic organisms.

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