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Comparison of milk oligosaccharides between goats with and without the genetic ability to synthesize α_{s1}-casein

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ABSTRACT

Milk oligosaccharides (OS)—free complex carbohydrates—confer unique health benefits to the nursing neonate. Though human digestive enzymes cannot degrade these sugars, they provide nourishment to specific commensal microbes and act as decoys to prevent the adhesion of pathogenic micro-organisms to gastrointestinal cells. At present, the limited quantities of human milk oligosaccharides (HMO) impede research on these molecules and their potential applications in functional food formulations. Considerable progress has been made in the study of OS structures; however, the synthetic pathways leading to their synthesis in the mammary gland are poorly understood. Recent studies show that complex OS with fucose and *N*-acetyl neuraminic acid (key structural elements of HMO bioactivity) exist in goat milk. Polymorphisms in the *CSN1S1* locus, which is responsible for synthesis of α_{s1}-casein, affect lipid and casein micelle structure in goat milk. The present study sought to determine whether *CSN1S1* polymorphisms also influence goat milk oligosaccharide (GMO) production and secretion. The GMO compositions of thirty-two goat milk samples, half of which were from genotype A/A (α_{s1}-casein producers) and half from genotype O/O (α_{s1}-casein non-producers), were determined with nanoflow liquid chromatography high-accuracy mass spectrometry. This study represents the most exhaustive characterization of GMO to date. A systematic and comprehensive GMO library was created, consolidating information available in the literature with the new findings. Nearly 30 GMO, 11 of which were novel, were confirmed *via* tandem mass spectrometric analyses. Six fucosylated OS were identified; 4 of these matched HMO compositions and three were identified for the first time in goat milk. Importantly, multivariate statistical analysis demonstrated that the OS profiles of the A/A and O/O genotype milks could be discriminated by the fucosylated OS. Quantitative analysis revealed that the goat milk samples contained 1.17 g/L of OS; however, their concentration in milks from A/A and O/O genotypes was not different. This study provides evidence of a genetic influence on specific OS biosynthesis but not total OS production. The presence of fucosylated GMO suggests that goat milk represents a potential source of bioactive milk OS suitable as a functional food ingredient.

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1. Introduction

Although milk evolved as the sole nourishment of the mammalian neonate, all known milks contain free oligosaccharides (OS) that are indigestible by infants (Engfer et al., 2000; Gnoth et al., 2000). Rather than functioning as a direct nutrient for the infant, human milk OS enhance the growth of specific bacteria and deflect the adhesion of pathogens in the infant gut (LoCascio et al., 2007; Morrow et al., 2005). Therefore, an HMO-like product would be valuable as a supplement for infant or even adult nutrition.

Goat milk has smaller casein micelles and fat globules, higher concentration of some whey proteins and oligosaccharides than bovine milk (Silanikove et al., 2010). Several studies report the effects of goat *CSN1S1* gene polymorphisms on milk production and composition, milk technological properties and on milk fatty acid content (Silanikove et al., 2010). Little is known, however, about the influence of these polymorphisms on milk oligosaccharides.

We were particularly interested in goat milk oligosaccharides (GMO) because of two studies, which suggested that GMO have anti-inflammatory effects and reduce intestinal inflammation in mice with induced colitis (Daddaoua et al., 2006; Lara-Villoslada et al., 2006). In addition, goat milk is known to contain fucosylated and sialylated OS, making them similar in composition to HMO (Chaturvedi and Sharma, 1990a; Urashima et al., 1994). Therefore, goat milk may represent an ideal source of OS for supplementary and therapeutic applications.

Recent advances in mass spectrometry have revealed the detailed structures of OS in mammalian milks. We employed high-throughput, high-resolution mass spectrometry to measure and identify OS from goat milk.

In addition to profiling the OS present in goat milk, we compared differences in OS production between goats of two different genotypes. α_{s1}-Casein is major protein component of milks (including goat milk) and makes up part of the well-known milk macrostructure, the casein micelle. The *CSN1S1* gene, which encodes for α_{s1}-casein, is highly polymorphic in goats, with 15 alleles: A, B_1, B_2, B_3, B_4, C, E, F, G, H, I, L, M, O1 and O2 characterized so far (Bevilacqua et al., 2002). These polymorphisms drive the large variability in the amount of α_{s1}-casein observed in individual goat milks (Moioli et al., 1998). The A/A genotype produces the most α_{s1}-casein (approximately ~7 g/L). A large deletion in the *CSN1S1* locus (called the O allele) precludes the expression of α_{s1}-casein, which is therefore absent in milk of goats of the O/O genotype. Inability to produce α_{s1}-casein, which is essential for casein micelle formation, results in the accumulation of large amounts of β- and κ-casein in the mammary epithelial cells' ER, and the micelles are not transported to the Golgi bodies properly (Chanat et al., 1999).

These major changes in casein micelle production, unsurprisingly, have a variety of effects on milk composition and quality. These effects have been intensively studied and include milk composition, renneting properties, cheese yield, and milk fat content (Grosclaude and Martin, 1997; Martin and Leroux, 2000). For example, A/A goats produce milk with higher protein and lipid content than O/O goats (Pierre et al., 1998).

The effects of these *CSN1S1* polymorphisms on OS synthesis remain uninvestigated. As OS synthesis occurs in the ER of the mammary epithelial cell (like α_{s1}-casein production), we hypothesized that the build-up of caseins in the O/O genotype would negatively impact the production and secretion of GMO. Therefore, MS was employed to profile OS in milks from both the A/A and O/O goat genotypes at the *CSN1S1* locus to see if the absence of α_{s1}-casein has any effect on OS synthesis. This study is the first to examine the effects of these polymorphisms on GMO synthesis.

2. Materials and methods

2.1. Sample collection and standards

Morning and evening milk samples were collected from 16 Alpine goats (8 from genotype A/A and 8 from genotype O/O, for a total of 32 milk samples) for identification and statistical comparison of neutral GMO. An additional set of 8 goat milk samples (4 of each genotype) were collected for analysis of GMO concentration and identification of acidic GMO. Three commercial goat milks were purchased at a local supermarket (Davis, CA, USA). Three samples of bovine milk were obtained from the University of California, Davis dairy barn. Three samples of human milk were obtained from a UC Davis lactation study directed by Dr. Jennifer Smilowitz. Monosaccharide standards—fucose (Fuc), *N*-acetylglucosamine (GlcNAc), galactose (Gal), glucose (Glc), *N*-acetylneuraminic acid (NeuAc), *N*-glycolylneuraminic acid (NeuGc), and D-allose—were purchased from Sigma–Aldrich (St. Louis, MO, USA). Maltopentaose, maltohexaose, and maltoheptaose were purchased from Sigma–Aldrich. The OS 3′-sialyl-lactose, 6′-sialyl-lactose, 3′-sialyl-*N*-acetyllactosamine, 6′-sialyl-*N*-acetyllactosamine, 3′-fucosyllactose, 2′-fucosyllactose, and lacto-*N*-neohexaose were purchased from Dextra (Reading, UK).

2.2. Oligosaccharide isolation and purification

OS were isolated from milk samples and purified according to a previously described method (Ninonuevo et al., 2006). Briefly, milk samples (0.5 mL) were diluted with an equal volume of nanopure water (18.2 MΩ ionic purity) and centrifuged at 4 °C for 30 min at 4000 × *g* to remove lipids. The fat-free fraction (infranate) was treated with 4 volumes of 2:1 (v/v) chloroform:methanol, and the emulsion was centrifuged at 4000 × *g* for 30 min at 4 °C. The lower chloroform and protein pellet were discarded. The upper layer containing OS was collected. Two volumes of pure ethanol were added to the OS fraction and the remaining protein was precipitated at 4 °C overnight. The samples were then centrifuged at 4000 × *g* for 30 min at 4 °C and the protein-free supernatant was collected and dried in a vacuum centrifuge at 37 °C. OS were rehydrated in 1 mL nanopure water. Residual peptides were removed using C8 columns (DSC-C8 Discovery, 3 mL tube capacity, 500 mg bed weight, Supelco, Bellefonte, PA, USA). The cartridges were conditioned with three column volumes (cv) of pure HPLC-grade acetonitrile (ACN) followed by three cv of nanopure water. The carbohydrate-rich solution was loaded onto the cartridge, and the peptide-free eluate was collected. The OS were further purified by nonporous graphitized carbon solid-phase extraction (GCC-SPE, 150 mg carbon, 4 mL tube capacity, Alltech, Deerfield, IL, USA). Prior to use, the GCC-SPE cartridge was activated with 3 cv of 80% ACN, 0.05% trifluoroacetic acid (TFA, v/v), and equilibrated with 3 cv nanopure water. The carbohydrate-rich solution was loaded onto the cartridge, and salts were removed by washing with three cv of nanopure water at a flow rate of 1 mL/min. For neutral OS profiling and statistical analysis, 32 milk samples were eluted from the cartridge using 2 cv of 20% ACN in water. For acidic OS profiling and total quantification, 8 milk samples were each eluted with 2 cv of 20% ACN in water followed by 2 cv of 40% ACN/0.1% TFA in water to collect the neutral and the acidic OS, respectively. Eluted fractions were dried prior to analysis by MS by vacuum centrifugation at 37 °C.