Original article

Sialylated oligosaccharides in mare and ass milk: preliminary results

Mario Orlandi[1], *Maria Claudia Curadi*[1], *Lucia Monti*[2], *Tiziana Maria Piera Cattaneo*[3]

[1] Department of Veterinary Science, Pisa University, Pisa, Italy - E-mail: maria.claudia.curadi@unipi.it; [2] Research Council for Agricolture and Economics - CREA, Lodi, Italy; [3] Research Council for Agricolture and Economics - CREA, Milano, Italy

Summary. In this preliminary investigation milk samples from 4 multiparous amiata asses and 5 half-breed mares belonging to the same farm located near Grosseto (Tuscany), feeding the same pasture, were collected and analyzed. Milk samples collection was performed in the first three mounths of lactation. Capillary electrophoresis (CE) analysis were performed to quantify 3-sialyllactose (3-SL) and 6-sialyllactose (6-SL). 3-SL and 6-SL highest amounts were found in mares samples (197.3 mg/L e 82.2 mg/L), while highest asses amounts were 45.1 mg/L and 49.8 mg/L respectively. 3-SL amounts were higher in all mares samples than in donkey samples; in mares samples 3-SL amounts were always higher than 6-SL.

Key words: mare milk, ass milk, oligosaccharides, 3-sialyllactose, 6-sialyllactose

Introduction

The term sialic acid (*sia*) refers to the different natural derivatives of neuraminic acid. They are electronegatively charged monosaccharides in higher animals and some microorganisms and they contribute to the enormous structural diversity of complex carbohydrates, which are major constituents mostly of proteins and lipids of cell membranes and secreted macromolecules (1). Sialyllactose is an oligosaccharide found in both human breast milk and cow's milk (2-4). It is the predominant sialylated component in milk (5, 6). Human milk contains high concentrations of complex carbohydrates, especially oligosaccharides which are shaped by extension of lactose through glycosyltransferase enzymes in the mammary gland. The most abundant structures are trisaccharides produced by addition of fucose or sialic acid to lactose. Sialylated oligosaccharides are more widely distributed whereas fucosylated oligosaccharides are missing from most mammalian milk (7-9). A function for non-immunoglobulins milk protective factors in providing for the defence of the nursling is only now beginning appreciated. Non-immunoglobulins are very active against whole classes of pathogens; such multifunctional antiviral agents include an α-2 macroglobulin like haemoagglutination inhibitor that could inhibit influenza and para-influenza viruses and a1 antitrypsin protects against rotavirus (10). Feeding infants breast milk of healthy mothers is associated with a lower incidence of infectious and allergic diseases. Although this effect is of multifactorial origin, it widely accepted that the entire intestinal flora of breast-fed infants provides antinfective properties and is an important stimulating factor for the postnatal development of the immune system (11). Some of the major milk oligosaccharides are α2,3-sialyllactose (3SL) and α2,6-sialyllactose (6SL), which are mainly produced by the sialyltranferases ST-3GAL4 and ST6GAL1, respectively. Milk oligosaccharides contribute also to the development of the intestinal environment with receptors for pathogens and as prebiotics, by promoting the colonization of the commensal bacteria. Analysis of intestinal microbiota showed in newborn mices different colonization patterns depending on the presence or absence of α2,3-sialyllactose in the milk (8, 12). Because milk oligosaccharides are neither digested nor absorbed in the small intestine they have been suggested to contribute to the development of the infant gastrointestinal tract and its colonization by commensal bacteria (13). Milk 3SL mainly affected the colonization of the intestine by *clostridial cluster IV* bacteria. This milk oligosaccharides prevent the adhesion of pathogenic bacteria to the epithelial surface in the gastrointestinal tract. For example α2,3-sialyllactose (3SL) binds the ulcerogenic bacteria Helicobacter pylori, decreasing the binding of the bacteria to duodenum-derived human cells. Fucosylated oligosaccharides prevent the binding of the major diarrhea-causing pathogens, including Campylobacter and major strains of caliciviruses to epithelial cells (12, 14, 15, 16, 17). Human milk oligosaccharides seem to promote the colonization of Bifidobacteria, which is found to be beneficial and major microbiome in the gut of breast-fed infants and show also anti-pathogenic effects protecting infants from different pathogenic microorganisms such as *Campylobacter jejuni, Campylobacter pylori, Escherichia coli, Vibrio cholera* and Rotavirus (18). High content of *sia* in mother milk, especially in colostrum where they are constantly found in milk ganglioside, shows that *sia* are important as infant nutrient (19). Recent data suggest that human milk oligosaccharides reduce HIV-1 binding to dendritic cell and reduce HIV-1 mother to child transmission (20). They influence the development of the intestinal microbiota by acting as selective nutrients, which support the proliferation of specific bacterial groups (7, 21, 22). The prebiotic action of milk oligosaccharides has been demonstrated by comparing the intestinal microbiota of infant fed on oligosaccarides rich breast milk and infant fed on formula (23). Furthermore, considering the structural similarity of milk oligosaccharides with cell surface glycan, milk oligosaccharides can function as soluble receptors, preventing the attachment of phatogenic bacteria to intestinal ephitelial cells (24, 25). Urashima *et al.* (26, 27) studied horse colostrum and characterized three neutral oligosaccharides. Nakamura *et al.* (28) studied also acidic oligosaccharides and found that 3-SL was the major component in horse colostrum along with Gal(β1-4) GlcNAc-1-phosphate, an unusual oligosaccharide that so far has not been found in the milk or colostrum of any other species, including humans, except for the cow (22). 6-SL was not detected in horse colostrum in that study (28). Since acidic milk oligosaccharides vary among domestic species (29), Albrecht *et al.* (30) made a comparative study to obtain a comprehensive overview of oligosaccharides present in the milk of different domestic animals. They evidenced that sialylated oligosaccharides account for approximately 80-90% of total oligosaccharides in the analyzed samples. Moreover, they found that total acidic oligosaccharide pool from (animal) milk was dominated by 3-SL/6-SL and 3-GL/6-GL. In particular, in mare colostrum, 3-SL was identified as preponderant component. 6-SL was evidenced, too, although on lower proportion. Currently, there is only a limited level of quantitative data on the total amount of oligosaccharides on individual components in animal milk, for exemple in cow milk, goat or buffalo milk (31, 32). Given similar deficiencies in equine milk, our first study considered the values of certain oligosaccharides in mare's milk compared with that of other species (33). A comparison of some milk oligosaccharides, only in colostrum, of different horse breeds was performed by Difilippo et al. (34), where differences were evident not only between breeds but also within every single breed. In the present investigation we wanted to evaluate 3SL and 6SL contents belonging to the samples from a group of mares and another of asses from the same breeding during the early periods of lactation.

Materials and Methods

The analyzed samples belong to milk of 4 multiparous amiata asses and 5 mares recognized previously free from infectious and parasitic diseases, reared in a farm located in the province of Grosseto, Italy. The collection was performed on the first (10 days after delivery), 2nd and 3rd month of lactation. Subjects were fed on permanent pasture hay produced by the farm whose composition was: 87 % dry matter, 9.6% crude protein, 3.2% fat , 28.8 % crude fiber (44.5% NDF, ADL 4.9%). All the animals had free access to pasture even from early spring pasture with the same chemical composition described above; during the observed lactation period an integration of commercial feed of 100g/bw with 11% crude protein and 4.2% crude fiber was also administered for every subject at the time of sample collection. In order to resolve and quantify 3-sialyllactose (3-SL) and 6-sialyllactose (6-SL) in milk, a CE technique using micellar electrokinetic chromatography (MEKC) was applied (35, 36). CE analyses were carried out by using a P/ACE MDQ Glycoprotein System (Beckman Coulter, Brea, CA, USA), with an uncoated capillary (ID = 50 mm; effective length = 50 cm). The run buffer solution was 55% aqueous (NaH_2PO_4 200 mM, pH 7.05, containing SDS 100 mM) and 45% methanol (MeOH). Sample injection was carried out by pressure at 0.7 psi for 6 sec and electrophoretic conditions were positive to negative (+30 kV) at 25°C. UV direct detection was at 200 nm. All samples were frozen at -20°C immediately after collection and thawed over-night at 4°C before analysis. Samples were defatted by centrifugation (microcentrifugette ALC 4214) at 5000*g* for 15 min and deproteinized: 400 μL of defatted milk were mixed with 800 μL ethanol and kept at 4°C overnight. The precipitated proteins were removed by centrifugation at 12000*g* for 10 min and the clear supernatant, containing oligosaccharides and lactose, was recovered and dried using a SpeedVac system (Thermo Fisher Scientific, Rodano (Mi), Italy). The dried residue was reconstituted with 100 μL of fresh 30% MeOH solution and analyzed. Commercial standards of 3-SL and 6-SL (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in 30% MeOH and used to create calibration curves and to spike samples to better identify oligosaccharides.

Results and Discussion

Values of 3-SL and 6-SL in mare and ass milk, obtained by CE analysis, are shown in Table 1.

Qualitative and quantitative variability in oligosaccharides composition has been evidenced both in human milk and milk from domestic animals, due to genetic variation and course of lactation (37-39). By the table's examination, although in few preliminary obtained samples, we observe that 3SL mare milk amounts seem to increase in the samples collected between first and third lactation month, while donkey samples seem to exhibit lower amount along the total observation period. Lower differences in 6SL samples amounts we note between mares and donkey. In our precedent work (40) we found higher 3SL mare's milk levels than donkey's, as in the present work, although

Table 1. 3-sialyllactose (3-SL) and 6-sialyllactose (6-SL) concentration in mare and ass milk at different sampling times

	3-SL (mg/L)			**6-SL (mg/L)**		
	Month of sampling			Month of sampling		
Mare	1	2	3	1	2	3
1	91.0	85.9	-	22.4	15.1	-
2	86.3	-	190.9	17.6	-	82.2
3	177.2	122.6	197.3	30.1	16.6	26.5
4	-	129.7	184.2	-	34.3	48.5
5	-	-	-	-	-	32.9
	3-SL (mg/L)			**6-SL (mg/L)**		
	Month of sampling			Month of sampling		
Ass	1	2	3	1	2	3
1	45.1	43.4	37.6	48.3	49.8	35.9
2	35.0	-	-	52.0	-	-
3	25.4	-	-	18.0	-	-
4	28.3	42.1	-	16.9	31.1	

always lower than in the present collection. 6 SL lower amounts we found in mare samples than in the present research. 3SL levels seemed always lower than in human milk, but not so far than goat's and cow's. 3SL mare's amounts found in the present research seemed higher than in goat's and cow's. Puente et al (41), found 4 time as much sialyc-acid in goat milk (230 mg/L) and in cow's (60 mg) and it seems that this content doesn't change according to the season. Other our researches about sialyloligosaccharides in milk of different species. Monti et al. (33) confirmed differences among animal species individuals and between stage of lactation. We observe also disialyllactose-N-tetraose (DSLNT) wich was higher than 3-SL and 6-SL and the most representative amount in human milk (455-805 mg/L). In most cases 3-SL showed to be the most concentrate of the quantified analytes with values ranging from 12 to 77 mg/L. Tao et al. (38) found that in bovine milk total oligosaccharides dropped rapidly in the first several days of lactation. Claps et al. (39) found significant differences in 3SL and 6SL amounts between milk samples from two breeds goat's as Mc Jarrow and Van Amelsfort-Shoonbeek (42) in bovine breeds. In both cases decrease was found during lactation.

Conclusions

The present investigation focalized the further important significance of sialylated oligosaccarides found both in ass and mare milk. Both 6-SL and 3-SL show high milk levels but it's interesting to observe higher 3-SL level in all mares milk samples at different lactatium period in consideration of the same nutrition level and quality. 3SL mare milk amounts seem to increase in the samples collected between first and third lactation mounth, while donkey samples exhibit lower amount than in mares in all collected samples. Lower differences in 6SL samples amounts we noted between mares and donkey. It will be interesting further investigations with more subjects and during longer observation period.

Technical and veterinary medical support were performed by Dr. Andrea Poggiaroni, whose important collaboration we sicerely regreat.

References

1. Schauer R. Sialic acids: fascinating sugars in higher animals and man. Zoology 2004; 107: 49-64.
2. Martin-Sosa S, Martin MJ, Garcia-Pardo LA, Hueso P. Sialyloligosaccharides in human and bovine milk and in infants formula: variations with the progression of lactation. J Diary Sci 2003, 86(1): 52-9.
3. Martin MJ, Martin-Sosa, Garcia-Pardo LA, Hueso P. Distribution of bovine milk sialoglycoconjugates during lactation. J Dairy Sci 2001; 84(5): 995-1000.
4. Wang B, Brand-Miller J, Mc Veagh P, Petocz P. Concentration and distribution of sialic acids in human milk and infant formula. Am J Clin Nutr 2001, 74 (4): 510-15.
5. Sieberg HC, Zang R, Scheidig A, et al. Interaction studies of sialicacids acids with model receptors contribute to nanomedicals therapies. J Neurol Disord 2015; 3: 212. Manca pagg
6. Shuai W, Grimm R, German JB, Lebrilla CB. Annotation and structural analysis of sialylated human milk oligosaccharides. J. Proteome Res 2011; 10(2): 856-68.
7. Urashima T, Saito T, Nakamura T, Messer M. Oligosaccharides of milk and colostrum in non-human mammals. Glycoconj J 2001, 18: 357-71.
8. Furher A, Sprenger N, Kurakevich E, Borsing L, Chassard C, Hennet T. Milk sialyllactose influences colitis in mice throught selective intestinal bacterial colonization. J of Experim Med 2010; 207(13): 2843-54.
9. Egge H. The diversity of oligosaccharides in human milk. In: New perspectives in infant nutrition. Renner and Sawatzki Ed, 1993.
10. Mc Lean BS, Holmes IH. Effect of antibodies trypsin and trypsin inhibitor on susceptibility of neonates to rotavirus infection. J Clin Microbiol 1981; 13:22-9.
11. Boehm G, Stahl B. Oligosaccharides from milk. J of Nutr 2007; 137(3): 847S- 49S.
12. Weiss GA, Hennet T. The role of sialyllactose in intestinal bacterial colonisation. Adv Nutr 2012; 3: 483-88.
13. Frank DN, Pace NR. Gastrointestinal microbiology enters the metagenomics era. Curr Opin Gastroenterol 2008, 24(1): 4-10.
14. Newburg DS. Oligosaccharides in human milk and bacterial colonization. J Ped Gastroenterol Nutr 2000; 30(2): S8-S17.
15. Newburg DS, Ruiz-Palacios GM, Altaye M et al. Innate protection conferred by fucosylated oligosaccharides of human milk against diarreha in breast fed infants. Glycobiology 2004; 14: 253-63.
16. Morrow AL, Rangel JM. Human milk protection against infectious diarrhea: implication for prevention and clinical care. Sem Pediatr Infect Dis 2004;15: 221-28.
17. Morrow AL, Ruiz-Palacios GM, Yiang X, Newburg DS. Human milk glycans that inhibit pathogens binding protect breast feeding infant against infectious diarrhea. J Nutr 2005;135(5): 1304-07.
18. Ferdouse A, Monirujjaman Md. Human milk oligosaccharides important for infant defence. N J Med Biol Sci 2014; 3 (1) 1-6.

19. Candra KP. Biological function of sialic acid production and their role in infant food industry. J. Tec. Pertanian 2008; 3(2): 50-7.
20. Hong P, Ninonuevo MR, Lee B, Lebrilla C, Bode L. Human milk oligosaccharides reduce HIV-1-gp120 binding to dendritic cell-specific ICAM3-grabbing non integrin (DC-SIGN). Br J Nutr 2009; 101(4): 482-86.
21. Uemura Y, Takahashi S, Senda A et al. Chemical characterization of milk oligosaccharides of a spotted Jena (Crocuta Crocuta). Comp Biochem and Physiol 2009; 152(2): 158-61.
22. Urashima T, Taufik E, Fukuda K, Asakuma S. Recent advances in studies on milk oligosaccharides of cows and other domestic farm animals. Biosci Biotech Biochem 2013; 77: 455-466.
23. Harmsen HJM, Wildeboerg-Veloo ACM, Rangs GC et al. Analysis of intestinal flora development in breast-fed and formula fed infants by using molecular identification and detection methods. J Ped Gastroenterol Nut 200; 30: 61-7.
24. Newburg DS. Neonatal protection by an innate immune system of human milk consisting of oligosaccharides and glycans. J An Sci 2009; 87(13): 26-34.
25. Bo L, Newburg DS. Human milk glycoproteins protect infants against human pathogens. Breast Feed Med. 2013; 8(4): 354-62.
26. Urashima T, Sakamoto T, Ariga H, Saito T. Structure determination of three neutral oligosaccharides obtained from horse colostrum. Carbohydrate Research 1989; 194, 280-87.
27. Urashima T, Saito T, Kimura T. Chemical structures of three neutral oligosaccharides obtained from horse (thoroughbred) colostrum. Comp Biochem Physiol B 1991; 100(1):177-83.
28. Nakamura T, Amikawa S, Harada T, Saito T, Araia I, Urashima T. Occurrence of an unusual phosphorylated N-acetyllactosamine in horse colostrum. Biochim et Biophys Acta 2001;1525, (1–2):13-8.
29. Nakamura T, Urashima T. The milk oligosaccharides of domestic farm animals. Trends in glycoscience and glycotechnology 2004; 16(88):135-42.
30. Albrecht S, Lane JA, Mariño K et al. A comparative study of free oligosaccharides in the milk of domestic animals. British J of Nutr 2014; 111: 1313-28.
31. Kunz C, Rudloff S. Health promoting aspects of milk oligosaccharides. Int Dairy J 2006; 16(11): 1341-46.
32. Mehra R, Kelly P. Milk oligosaccharides: structural and technological aspects. Int. Dairy J 2006; 16(11): 1334-40.
33. Monti L, Curadi MC, Orlandi M, Cattaneo TMP. Capillary electrophoresis analysis of sialyloligosaccharides in milk of different species. It J Anim Sci 2011;10:99.
34. Difilippo E, Willems HAM, Vendrig JC, Fink-Gremmels J, Gruppen H, Schols HA. Comparison of milk oligosaccharides pattern in colostrum of different horse breeds. J. Agric Food Chem 2015; 63 (19): 4805-14.
35. Bao Y, Zhu L, Newburg DS. Quantification of sialyloligosaccharides from human milk by capillary electrophoresis. Analyt Biochem 2007; 370: 206-14.
36. Bao Y, Newburg DS. Capillary electrophoresis of acidic oligosaccharides from human milk. Electrophoresis 2008; 29: 2508-15.
37. Coppa GV, Pierani P, Zampini L, Carloni I, Carlucci A, Gabrielli O. Oligosaccharides in human milk during different phases of lactation. Acta Pædiatr Suppl 1999; 430: 89-94.
38. Tao N, DePeters EJ, German JB, Grimm R, Lebrilla CB. Variations in bovine milk oligosaccharides during early and middle lactation stages analyzed by high-performance liquid chromatography-chip/mass spectrometry. J Dairy Sci 2009; 92: 2991-3001.
39. Claps S, Di Napoli MA., Sepe L et al. Sialyloligosaccharides content in colostrum and milk of two goat breeds. Small Ruminant Research 2014; 121: 116-9.
40. Monti L, Cattaneo TMP, Orlandi M, Curadi MC. Capillary electrophoresis of sialylated oligosaccharides in milk from different species. J Cromatogr A 2015; 1409: 288-91.
41. Puente R, Garcia-Pardo LA, Rueda R, Gil A, Hueso P. Seasonal variation in the concentration of gangliosides and sialic acids in milk from different mammalian species. Int Diairy J 1996; 6 (3): 315-22.
42. Mc Jarrow P, Van Amelsfort-Schoonbeek J. Bovine sialyloligosaccharides: seasonal variation in their concentration in milk and a comparision of the colostrums of Jersey and Fresian cows. Int Dairy J 2004; 14: 571-9.

Correspondence:
Maria Claudia Curadi
Department of Veterinary Science
Pisa University,
Viale Piagge, 2
56123 Pisa, Italy
E-mail: maria.claudia.curadi@unipi.it