Profile and content of sialylated oligosaccharides in donkey milk at early lactation

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Abstract

Animal milk oligosaccharides can be used as bioactive ingredients in functional foods and infant milk formulas. Donkey milk is already considered a functional food and a possible substitute for human milk. The aim of this study was to identify the structure and abundance of sialylated milk oligosaccharides from five Amiata donkeys during four different stages of early lactation. Liquid chromatography combined with mass spectrometry analysis led to the identification and quantification of seven sialylated oligosaccharides: 3-sialyllactose, 6-sialyllactose, sialyllacto-N-tetraose a, sialyllacto-N-tetraose b, sialyllacto-N-tetraose c, Neu5Ac(α2–3) + Gal(β1–4)GlcNAc(β1–6)(Gal(β1–3))Gal(β1–4)Glc and Neu5Ac(α2–6) + Gal(β1–4)GlcNAc(β1–6)(Gal(β1–3))Gal(β1–

4)Glc. The latter five oligosaccharides were identified in donkey milk for the first time. The most abundant sialylated oligosaccharide found was 6-sialyllactose. In addition, variations in the content of four oligosaccharides were highlighted over the experimental period: the highest values were found at 15 days of lactation, and the lowest at 60 days.

Keywords: donkey milk, milk oligosaccharides, sialylated milk oligosaccharides, milk quality, nutraceutical.

1. Introduction

In all mammals, milk represents food for new-borns and contains both nutritive and protective components. The main nutritional components of milk are represented by protein, lipids, lactose, minerals and vitamins. These milk nutrients are essential to support the development of the newborn until the weaning period (Pereira, 2014). Milk oligosaccharides are a complex class of carbohydrates that provide no direct nutritional value to the offspring but act as a bioactive factor in numerous defensive and physiological functions (Doherty et al., 2018). Oligosaccharides are present in milk as free molecules or conjugated with other components (Ranjan et al., 2016). In human milk, oligosaccharides serve as a substrate for beneficial microbiota, by acting as prebiotics, and inhibit the intestinal adhesion of pathogenic microorganisms, thus limiting the onset of enteric infections (Smilowitz, Lebrilla, Mills, German & Freeman, 2014). Human milk oligosaccharides also show a positive effect on immune system development (Plaza-Díaz, Fontana & Gil, 2018), allergic disorders (Doherty et al., 2018), and autoimmune diseases (Xiao et al., 2018), as well as contributing to brain development and cognition (Bode, 2012). Since milk oligosaccharides show specific beneficial effects on health, they can be defined as functional components and nutraceuticals, according to the definition reported by Santini et al. (2018) and Santini & Novellino (2018), respectively. Milk oligosaccharides are synthesized in the mammal gland starting from five different monosaccharides:

51 D-glucose, D-galactose, N-acetylglucosamine, L-fucose and N-acetylneuraminic acid (NeuAc, also
52 known as sialic acid) (Oliveira, Wilbey, Grandison, & Roseiro, 2015). These monosaccharide units
53 are commonly combined with a lactose core through numerous possible linkages, resulting in a wide
54 range of different structures that reflect the multitude of their biological functions. Human milk
55 oligosaccharides have been extensively investigated and more than 200 different oligosaccharides
56 have been identified, including many isomers (Sischo, Short, Geissler, Bunyatratchata, & Barile,
57 2017). On the other hand, farm animal milk oligosaccharides have been studied less. However,
58 between 35-48 oligosaccharides have been characterized in cow, goat, sheep, pig, horse and camel
59 milk (Albrecht et al., 2014). Milk oligosaccharides are usually classified in two main classes: neutral
60 (with no charge) and acidic (with a negative charge, if sialylated). In human milk, neutral
61 oligosaccharides are the most represented, while in animal milk, sialylated oligosaccharides account
62 for about 80-90% of total oligosaccharides (Albrecht et al., 2014). However, Karav, Salcedo, Frese
63 & Barile (2018) reported that in mare milk the neutral oligosaccharides were the predominant. In
64 human milk, oligosaccharides are one of the most represented solid components and mature milk
65 contains about 7-12 g/L of oligosaccharides. In contrast, the concentration of oligosaccharides in the
66 milk of traditional dairy animals is 10-100 times lower (Boehm & Stahl, 2007). In particular, cow
67 milk contains 0.03-0.06 g/L (Kelly et al., 2013) and mare milk about 0.08-0.22 g/L of
68 oligosaccharides in the first week of lactation (Karav et al., 2018). In human milk, variations in the
69 oligosaccharides content have been reported among individuals, during lactation and according to
70 blood group (Bode, 2012). In cow milk, the content and composition of milk oligosaccharides vary
71 among breeds, individuals and over lactation (Tao, DePeters, German, Grimm, & Lebrilla, 2009). In
72 recent years, the scientific and industrial focus on animal milk oligosaccharides has increased, since
73 they can be used as bioactive ingredients in functional foods and infant milk formulas. Donkey milk
74 is already considered a functional food and a promising substitute for human milk when mothers
75 cannot breast feed and in particular when infants are allergic to cow milk protein (Martini, Altomonte,

Licitra, & Salari, 2018). In fact, donkey milk has been used over centuries to feed orphan children and today has been proved to be the closest to human milk, above all in terms of lactose, protein and ash content (Altomonte, Salari, Licitra, & Martini, 2019). Compared to cow milk, both human and donkey milk are characterized by high lactose content and low protein, casein and ash content. In addition, both the protein profile and the digestibility of the protein fractions of human and donkey milk is similar, whereas in cow milk the higher content of caseins make it less digestible for the human infant (Martini et al., 2018). Donkey milk was also recently used as an ingredient for developing a milk fortifier for the nutrition of preterm infants and was shown to have better feeding tolerance results compared to a commercial bovine milk derived fortifier (Bertino et al., 2019).

Research on donkey milk oligosaccharides in very limited. To date, only six oligosaccharides have been identified in donkey milk and no variability factors have ever been evaluated (Monti, Cattaneo, Orlandi, & Curadi, 2015; Ranjan et al., 2016; Singh, Maurya, Rizvi, & Deepack, 2017). Recently, Yan et al. (2018) highlighted that donkey is the only species (among eight farm animals) to show similar 3-sialyllactose/6-sialyllactose patterns as human milk, with more 6-sialyllactose than 3-sialyllactose. However, research on animal models has already shown that donkey milk oligosaccharides are able to stimulate specific and non-specific immunological resistance (Ranjan et al., 2016). In Italy, one of the main asinine breeds reared for milk production is the Amiata donkey (Altomonte, 2019). In this breed, the lactation period lasts, on average, 300 days (Martini, Altomonte, Salari, & Caroli, 2014; Martini, Altomonte, Manica & Salari, 2015). Given the growing interest in donkey milk in pediatrics and the importance of oligosaccharides as bioactive ingredients in functional foods and infant milk formulas, our aim was to identify the structure and abundance of sialylated milk oligosaccharides in donkeys. The goal of this study was also to verify if asinine milk can be a valid source of oligosaccharides; for this reason, for the first time, the effect of lactation stage on the identified milk oligosaccharides content was evaluated.

2. Material and Methods

2.1 Animals and samplings

The milk from a single herd of Amiata breed donkeys was collected on a Tuscan farm (central Italy) that produces and sells milk for human consumption in accordance with the requirements of Regulation (EC) No 853/2004. Five healthy pluriparous (parity 4.20±1.64), aged 11.20±5.76 years and with a body weight of 296±13 kg (mean±standard deviation) were selected for the study. Jennies were reared in a semi-intensive system and were all fed with grass hay ad libitum plus 2kg per day per head of commercial concentrate. Animals had free access to clean and fresh water. Jennies were machine-milked twice per day, and the foals were separated from their mothers three hours before milkings. At each milking, milk yield was recorded using a lactometer connected to the milking machine. Milk samples were collected at four different lactation stages: 15, 30, 45 and 60 days, for a total of twenty individual samples. Each sample was obtained by mixing the two individual daily milkings, and for each sample, two aliquots were prepared. Overall, twenty aliquots were refrigerated at 4 °C and analysed in relation to gross chemical composition within 24 hours. The other twenty samples were immediately stored at -80 °C after collection, then freeze-dried and stored at -20 °C until oligosaccharides analysis. A flow diagram of sampling procedures was presented in Figure 1. Freeze-drying was performed in the knowledge that donkey milk chemical parameters are not affected by this treatment (Polidori, Spera, Sabatini, & Vincenzetti, 2019). All the analysis were performed in duplicate.

Figure 1

2.2 Analysis of chemical milk composition

A total of 240 analyses were carried out. The following parameters were evaluated for each fresh donkey milk sample: proteins, caseins, fat and lactose contents by infrared analysis using a fully

126 automatic milk analyser (MilkoScan™ 7 RM; Italian Foss Electric, Padua, Italy) (details are provided
127 in the supplementary material); ash content (defined as inorganic substances remaining in the residue
128 after ignition) was determined using a muffle furnace operating at 550 ºC according to the methods
129 of the Association of Official Analytical Chemists (AOAC, 1990) and pH by potentiometric method
130 using a Thermo Fisher Scientific Inc. pHmeter (Waltham, USA).
131
132 **2.3 Analysis of milk oligosaccharides**
133 Dimethylsulfoxide (DMSO), 2-aminobenzamide (2-AB), sodium cyanoborohydride, acetic acid and
134 ethanol were obtained from Sigma-Aldrich (Saint Louis, USA). Water (18.2 MΩ) was generated by
135 an ELGA ultrapure water system of Veolia Water Technologies (Birmingham, UK). 3-sialyllactose
136 standard was prepared at the Dalian Institute of Chemical Physics laboratory (Dalian, China). Briefly,
137 1 mL of water was added to each freeze-dried milk sample (150 mg) and the mixture was then
138 centrifuged at 8500 rpm for 10 minutes at 4 °C to remove lipids. The defatted milk sample (500μL)
139 was mixed with 1 mL of pure ethanol and stored overnight at 4 °C. Subsequently, the protein
140 precipitate was separated by centrifugation at 8500 rpm for 10 minutes at 4 °C and the clear
141 supernatant (1 mL), mainly containing lactose and oligosaccharides, was collected and evaporated to
142 dryness using a centrifugal concentrator at 10 °C.
143 Oligosaccharides were fluorescently labelled with 2-AB following Bigge et al. (1995). Briefly, the
144 dried extract was dissolved with 200 μL of the 2-AB labelling reagent (DMSO/acetic acid (70/30)
145 containing 1.05 M of 2-AB and 3 M of sodium cyanoborohydride). The reaction mixture was
146 incubated at 65 °C for 2 hours and then was used for analysis.
147 The liquid chromatography (LC) analysis was performed with an Ultimate 3000-RS ultra high
148 performance liquid chromatography (UHPLC) system equipped with an RF-2000 fluorimeter (FLD)
149 and a 2-way 10 port high pressure switching valve (all from Thermo Fisher Scientific, Waltham,
150 USA). LC separation was performed using our previous analysis method (Yan et al., 2018). Detection

151 was performed using excitation wavelength 330 nm and emission wavelength 420 nm. X500B QTOF
152 (AB Sciex, USA) mass spectrometry (MS) was combined and used to identify oligosaccharides. The
153 source gas temperature was 400 °C with Ion gas 1: 45psi, Ion gas 2: 50psi and Curtain gas: 35psi.
154 Both MS spectra were acquired in the positive-ion mode with an acquisition rate of 1 second per
155 spectrum over a mass range of m/z 300-2000. Donkey milk oligosaccharides identification and
156 quantitation were performed via ABSCIEX SCIEX OS Analysis software (version SCIEX OS 1.5).
157 We used 3-sialyllactose as standard for the quantification of the other sialylated oligosaccharides.
158 After 3-sialyllactose was derivatized with 2-AB, a quantitation curve was prepared and a linear
159 regression equation ($y = 178127x - 61680$) was obtained. Excellent linearity (R^2>0.9999 for over 0.1-
160 102.4 µg/mL) was obtained. The standard curve was then used for the quantitative analysis of all
161 derivatized sialylated oligosaccharides in donkey milk. In total, 40 samples were analysed.
162
163 **2.4 Statistical analysis**
164 The results of the milk yield, milk gross composition and sialylated oligosaccharide content were
165 analysed using ANOVA for repeated measurements, considering the lactation period as the fixed
166 effect and the subject as a random effect. Least significance means were compared by the t-test.
167 Significant differences were considered at $P \leq 0.01$ or $P \leq 0.05$. Statistical analysis was carried out using
168 JMP software (SAS Institute, 2002).
169
170 **3. Results and discussion**
171 Table 1 shows Amiata donkey milk yield and composition during the experimental period.
172
173 **Table 1**
174

175 The daily milk yield was calculated as a sum of the two daily milkings which during the first two
176 months of lactation was on average 1363.16±275.70 mL, in line with Alabiso, Giosuè, Alicata,
177 Mazza, & Iannolino (2009) who have reported a milk yield of 1.18 kg using the same number of daily
178 milkings and the same milking interval. In addition, the milk yield was constant throughout the
179 experimental period, despite a generally higher quantity at 30 days. The pH of donkey milk showed
180 an average value of 7.10±0.06 and no significant changes occurred during the examined lactation.
181 This is in agreement with Guo et al. (2007), who reported that pH values in donkey milk range from
182 7 to 7.2 and are not influenced either by breed or stage of lactation. In our study, a significant decrease
183 in protein and ash contents was observed during the experimental period, according to another study
184 on donkey milk (Guo et al., 2007). In fact, as reported by Ozturkoglu-Budak (2018), the lactation
185 stage significantly influenced the protein and ash content of donkey milk. Regarding the casein
186 content, our findings were in line with Giosuè, Alabiso, Russo, Alicata, & Torrisi (2008) who reported
187 a decreasing trend in the casein content during lactation. The fat and lactose content did not undergo
188 significant variations during the first 60 days of lactation, in agreement with Martini et al. (2014).
189
190 Seven sialylated oligosaccharides were identified and quantified in this study: 3-sialyllactose (3-SL);
191 6-sialyllactose (6-SL); sialyllacto-N-tetraose a (LSTa); sialyllacto-N-tetraose b (LSTb); sialyllacto-
192 N-tetraose c (LSTc); Neu5Ac(α2–3) + Gal(β1–4)GlcNAc(β1–6)(Gal(β1–3))Gal(β1–4)Glc (3-SLNP)
193 and Neu5Ac(α2–6) + Gal(β 1–4)GlcNAc(β1–6)(Gal(β1–3))Gal(β1–4)Glc (6-SLNP).
194 While 3-SL and 6-SL have already been reported in donkey milk, the other five were found for the
195 first time in asinine milk. However, LSTa and LSTb were found only in two individuals and were not
196 subjected to statistical analysis. The other oligosaccharides were found in all the animals and at each
197 lactation period, and the results are presented in Table 2.
198
199 **Table 2.**

200

201 A chromatogram of a donkey milk sample is reported in Figure 2.

202

203 **Figure 2**

204

205 The most abundant sialylated oligosaccharide found was 6-SL, in line with Monti et al. (2015) and
206 Orlandi, Curadi, Monti, & Cattaneo (2016). All the oligosaccharides quantified show the highest
207 content at 15 days and the lowest content at 60 days of lactation. Similarly, the content of sialylated
208 oligosaccharides decline over lactation in cow and porcine milk (Tao et al., 2009; Tao, Ochonicky,
209 German, Donovan, & Lebrilla, 2010) and during the first week of lactation in mare milk (Karav et
210 al., 2018). Instead, in human milk the sialylated oligosaccharides content remains relatively constant
211 during lactation (Niñonuevo et al., 2008). On average, the 3-SL content (10.26±4.01) was lower,
212 while the 6-SL content (18.99±4.02) was similar to the findings reported by Monti et al. (2015),
213 however no information on donkey breed and lactation stage of the animals were reported in their
214 study. In a subsequent study on Amiata donkeys at early lactation, Orlandi et al. (2016) reported
215 higher individual contents for both 3-SL and 6-SL, with values ranging from 25.4 to 45.1 mg/L for
216 3-SL and from 16.9 to 52.0 mg/L for 6-SL. The differences found in these preliminary studies may
217 be due to the sampling design.
218 In agreement with the results of Yan et al. (2018) in donkey milk, we found more 6-SL than 3-SL.
219 The mean 3-SL content in donkey milk was lower than cow milk (47 mg/L), where 3-SL is the
220 predominant oligosaccharide (Fong, Ma, & McJarrow, 2011), mare milk (on average 85 mg/L in the
221 first week of lactation) (Karav et al., 2018) and human milk (198-259 mg/L) (Sprenger, Lee, De
222 Castro, Steenhout, & Thakkar, 2017). A significant decrease in 3-SL was highlighted throughout the
223 lactation period examined, as occurs in mare milk (Karav et al., 2018). However, 3-SL did not vary
224 significantly during lactation in cow and human milk (Martín-Sosa, Martín, García-Pardo, & Hueso,

2003; Sprenger et al., 2017). The mean 6-SL content in donkey milk was higher than cow milk (3.6 mg/L) (Fong et al., 2011) and mare milk (15.1 mg/L) (Karav et al., 2018), but lower than human milk (120-561 mg/L) (Sprenger et al., 2017). The significant reduction in 6-SL content over the observed lactation in donkey milk is in line with the results reported in both human and cow milk (Martín-Sosa et al., 2003; Sprenger et al., 2017). In mare milk, 6-SL content initially increases and then decreases during the first week of lactation (Karav et al., 2018). Both 3-SL and 6-SL are well-studied oligosaccharides and they show anti-inflammatory properties, promote the growth of beneficial gut bacteria, play an important role for brain development and for cognitive functions and diminish stressor-induced anxiety-like behavior (Tarr et al., 2015). Moreover, 3-SL supports immune homeostasis and protects against osteoarthritic development (Jeon et al., 2018). LSTa was found only in one donkey at 60 days of lactation (1.44 mg/L) and LSTb only in five samples: at every sampling in one donkey (with an increasing trend from 0.4 mg/L at 15 days to 1.76 mg/L at 60 days of lactation), and just at 60 days of lactation in another donkey (1.10 mg/L). The mean LSTc content (0.49±0.33) showed a significant decrease from 15 days to 45-60 days of lactation. LSTa, LSTb and LSTc were found in higher concentrations in human milk (more than 1 g/L) with a decreasing trend over lactation, and, to our knowledge were not detected in both cow and mare milk (Martín-Sosa et al., 2003). However, LSTc was found in Bactrian camel and reindeer milk (Fukuda et al., 2010; Taufik et al., 2014). LST variants shown to play a role in the modulation of intestinal epithelial cell maturation and exhibit antimicrobial and antibiofilm activities (Craft, Thomas & Townsend, 2019). The mean 3-SLNP content (0.42±0.17) was significantly higher at 15 days, while in the remaining lactation stages, it was low and constant. Finally, the mean 6-SLNP content (0.27±0.10) did not show significant changes during lactation. Both 3-SLNP and 6-SLNP were found in cow milk but were not quantified, little information is available in the literature regarding 3-SLNP and 6-SLNP in human milk (Albrecht et al., 2014; Urashima, Messer & Oftedal, 2017) and, to our knowledge, were not identified in mare milk and their physiological function are still not investigated. Overall, our results

on donkey milk oligosaccharide variations over lactation are similar to a study carried out on porcine milk, where it was observed that the content of all major oligosaccharides decrease significantly throughout lactation, while the content of some minor milk oligosaccharides remains relatively unchanged or increase at late lactation (Tao et al., 2010). Unlike Monti et al. (2015), we did not detect the sialylated oligosaccharides disialyl-lacto-N-tetraose (DSLNT).

4. Conclusions

In this study, seven sialylated oligosaccharides were identified and quantified in donkey milk, five of which were found for the first time. In addition, despite the quite short lactation period studied, this research has allowed to highlight significant variations in the content of four oligosaccharides. Further studies using other breeds and for longer lactation period are needed in order to expand the knowledge on sialylated oligosaccharides of donkey milk. Moreover, even the neutral oligosaccharides of donkey milk need to be studied. In conclusion, donkey milk is a promising source of sialylated oligosaccharides and in particular of 6-SL and could be used by the industry as an ingredient for developing innovative baby formula milks or functional foods.

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Declarations of interest

The authors declare that there is no conflict of interest.

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