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CHAPTER

31

Nutritional Value and Potential Health Benefits of Donkey Milk

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INTRODUCTION

Milk of each mammal is specifically designed to provide perinatal passive immunization (colostrum and transitional milk) and fulfill the nutritional requirements of the newborn. Human breast milk is certainly the best food for infants, at least for the first 4 months of life, although there may be several cases (lack of milk ejection, orphans, or sick mothers) for which it is necessary to find a valid alternative. Cows are the primary dairy animal species throughout the world because of the abundance of their lacteal secretion, needed to satisfy the demand of milk and dairy products for human nutrition. However, cow milk is not suitable for the infants affected by cow milk protein allergies (CMPA), the prevalence for which was estimated between 5% and 15%, including infants who show symptoms related to adverse reactions to cow milk protein (Vandenplas et al., 2007). Severe sensitivity of these subjects was also occasionally detected in clinical cross-reactivity between milks of other ruminants (Järvinen and Chatchatee, 2009). Thus, nonbovine milks from other domesticated dairy species have recently gained economic value for their high quality as an alternative food and especially for medical purposes. The latter case prompted the investigations on donkey milk, which was found to have adequate nourishment and is well tolerated by children with CMPA in terms of clinical tolerability (Monti et al., 2007). The hypoallergenic properties, together with other peculiar and health-promoting aspects reported by many authors, initiated great interest in donkey milk as confirmed by the increased number of articles published in the last few years.

Main characteristics and nutritional value, with the related impact on human health and some potential applications of donkey milk in the dairy field, are reviewed in this chapter.

CHARACTERISTICS OF DONKEY MILK

In the last 10 years the number of donkeys in existence for breeding purposes has constantly increased in some European countries; however, the availability of donkey milk is still limited, primarily by the low milk production. Recent studies on donkey lactation curves showed that individual milk yield ranged between 1.54 and 1.73 kg/day on specialized farms (Bordonaro et al., 2013), which generally raise animals in semiextensive conditions and care about their wellness. Equid mammary gland has a low average capacity (maximum 2.5 L); therefore, to increase milk supply, dairy equids may need to be milked many times a day (Salimei and Fantuz, 2012). Currently, most donkey milk is sold directly from the producers, raw or pasteurized, or freeze-dried at pharmacy stores.

Comparing the gross composition of donkey and human milk, the most apparent difference is certainly the fat content, which is much lower in donkey milk (Table 31.1). Differently, the other main components of donkey milk, such as lactose, caseins, and whey proteins, are very similar to human milk, whereas they remarkably differ from cow milk. This high similarity might generally explain the high tolerance of donkey milk shown by humans (Guo et al., 2007).

TABLE 31.1 Gross Composition of Donkey, Human, and Cow Milk

	Donkey Milk	Human Milk	Cow Milk
pH	7.0–7.2	7.0–7.5	6.6–6.8
Protein (g/100 g)	1.3–1.8	0.9–1.7	3.1–3.8
Fat (g/100 g)	0.3–1.8	3.5–4.0	3.5–3.9
Lactose (g/100 g)	5.8–7.4	6.3–7.0	4.4–4.9
Caseins (g/100 g)	0.6–1.0	0.3–0.4	2.4–2.8
Whey proteins (g/100 g)	0.4–0.9	0.6–0.8	0.5–0.7
Energy (kj/kg)	1719	2680	2883

The pH of donkey milk, as well as human milk, is neutral or slightly alkaline, likely due to low content of caseins and phosphates, in comparison to cow milk. The high lactose content makes this milk sweet and palatable, resulting in it being well accepted by children. As for the protein distribution, caseins represent generally around 50% of total proteins and four different protein fractions have been identified (α_{s1}-, α_{s2}-, β-, and *k*-casein) so far. The two major caseins of donkey milk are α_{s1}- and β-casein, given that α_{s2}-casein was found only as a minor component, whereas *k*-casein was rarely detected by means of a specific immunostaining method (Chianese et al., 2010). Whey proteins are principally constituted by α-lactoalbumin, β-lactoglobulins, and lysozyme together with minor components such as serum albumin and lactoferrin (Cunsolo et al., 2007). Although the relative amount of the whey proteins showed a decreasing trend throughout lactation, the most abundant components are always α-lactoalbumin (2240–3090 mg/L) and lysozyme (1040–2970 mg/L) (Gubić et al., 2016). The relevant concentration of lysozyme, much higher than cow milk (<0.013 mg/L) and human milk itself (43–81 mg/L), is a peculiar characteristic of donkey milk, and it is a trait extensively studied by researchers (Król et al., 2010; Gubić et al., 2015). A lower contribution is given by the lactoglobulins fraction (200–260 mg/L), absent in human milk (Gubić et al., 2015). The lactoglobulins in donkey milk are constituted by two monomeric components, namely β-lactoglobulin I, which displays a high structural similarity with the horse lactoglobulin, and β-lactoglobulin II, which shows substantial differences in the sequence with the horse counterpart (Godovac-Zimmermann et al., 1990). Immunoglobulins (<88.3 mg/L) and lactoferrin (13–87 mg/L), which also tend to drop during lactation, are minor protective components that confirm their beneficial impact on gut health and immune defense system at an early and middle lactation (Gubić et al., 2016).

Donkey milk fat globules showed an average diameter of 2 μm for 70% of total globules, resulting similar to the horse ones but smaller than both human (4 μm) and cow (2.8–4.6 μm) fat globules (Claeys et al., 2014; Martini et al., 2013). Moreover, equine milk does not cream due to the lack of cryoglobulin, a protein that adsorbs onto the fat globules as the temperature is reduced, and thus the agglutination of fat globules occurs very slowly (O'Mahony and Fox, 2014). Within a diet exclusively based on donkey milk, the low fat and energetic content (Table 31.1) could be a limiting factor for a sufficient weight gain in infant nutrition. This was the reason why the addition of safe oils, such as olive or sunflower oil, was suggested to reach a suitable energetic intake for infants (Swar, 2011; Tidona et al., 2015). Nevertheless, the composition of donkey milk fat reveals an interesting qualitative profile, because it consists of 80%–85% of triglycerides, and higher levels of free fatty acids (9.5%) and phospholipids (5%–10%) compared to human milk (0.5%–1.5% and 0.7%–1.5%, respectively) (Claeys et al., 2014). Among the saturated fatty acids, palmitic acid (19.94% average) was observed to be dominant, whereas the unsaturated fatty acids totally accounted for 48.02%, composed by 28.00% of mono- (MUFA) and 20.02% of polyunsaturated (PUFA) fatty acids (Martemucci and D'Alessandro, 2012). Oleic acid (21.50% average) is the most representative of MUFA, whereas the content of PUFA mainly consists of α-linolenic and linoleic acid. The high levels of PUFA in donkey milk, compared to other monogastric herbivores, may indicate the absence of hydrogenation of fatty acids in the digestive tract before absorption, which occurs in ruminants (Jenkins et al., 1996).

The content of most minerals is higher in donkey milk than in human milk, but significantly lower than in ruminant milk (Fantuz et al., 2012). The vitamin content of donkey milk is generally comparable or slightly lower than human milk, and, on average, lower than the vitamin content of ruminant milk, except for the vitamin C level (Claeys et al., 2014).

MICROBIOLOGICAL CHARACTERISTICS AND HYGIENIC ASPECTS

Although data on the potential health benefits and chemical composition of donkey milk increased considerably over the last 10 years, information on microbiological and safety characteristics have less availability. A recent survey on donkey milk collected in farms of Northwestern Italy showed significant variations of the microbiological content related to the different milking practices adopted (Cavallarin et al., 2015). The overall mean of total viable count (TVC) was 5.38 log colony-forming units (CFUs)/mL, with the lowest TVC values (i.e., 2.84 log CFUs/mL) in the milk coming from the farm where the animals were free to pasture and were hand-milked. Other studies reported TVC values generally one order of magnitude lower (≤4 log CFUs/mL) (Coppola et al., 2002; Salimei et al., 2004; Zhang et al., 2008; Sarno et al., 2012; Malissiova et al., 2016; Tidona et al., 2015; Aspri et al., 2017). In a study on mammary gland health status and milking hygiene (Pilla et al., 2010), milk samples showed very low TVC values (<2 log CFUs/mL), detecting few pathogens when hygienic milking procedures were applied. The only pathogenic bacteria isolated, i.e., *Staphylococcus aureus* and streptococci pathogenic, for equids such as *Streptococcus equi* and *Streptococcus equisimilis*, represented 6%, 2%, and 1% of the isolates, respectively. The presence of *Pseudomonas* spp. and *Enterobacteriaceae* in raw donkey milk highlighted the importance of animal management and good dairy farming practices (Sarno et al., 2012; Cavallarin et al., 2015; Giacometti et al., 2016). The search for human pathogens, such as *Salmonella* spp., *Escherichia coli*, *E. coli* O157, *Listeria monocytogenes*, *Bacillus cereus*, and *Campylobacter* spp., in Italian, Greek, and Cypriot donkey milk samples gave negative results (Sarno et al., 2012; Cavallarin et al., 2015; Malissiova et al., 2016; Aspri et al., 2017). Only *B. cereus* was found in raw donkey milk until levels of 3 log CFUs/mL, and it was also detected in pasteurized donkey milk stored at 4 and 12°C with values ranging between 1 and 7 log CFUs/mL, according to storage conditions (Scatassa et al., 2011; Cavallarin et al., 2015; Giacometti et al., 2016). The presence of *B. cereus* spores and the capacity of vegetative cells to multiply represent a key point for safety and a critical point to be investigated. The low microbial content and the rare presence of pathogenic bacteria were ascribed to the natural antimicrobial substances, mainly lysozyme and lactoferrin, present in donkey milk. Furthermore, the mammary gland status of dairy donkey is generally healthy. Mastitis is rare, mainly of traumatic origin, and does not represent a limiting factor in milk production (Salimei and Fantuz, 2012). The implementation of hygienic practices and regulations, such as pasteurization and Hazard Analysis and Critical Control Point (HACCP) plans, should further improve the production of donkey milk of high quality and safety (Zhang et al., 2008). With regard to the microbiota of donkey milk, the lactic acid bacteria (LAB) represent the major portion. The LAB usually reaches counts of 3–4 log CFUs/mL, representing nearly 80% of the total microbial content (Zhang et al., 2008; Carminati et al., 2014; Cavallarin et al., 2015). LAB counts decreased during cold storage of donkey milk, and this was attributed to a possible antimicrobial effect of lysozymes that inhibit the LAB development (Salerno et al., 2011; Cavallarin et al., 2015). Conversely, the relatively good growth of indigenous LAB during storage of donkey milk at 20°C suggested that the natural antimicrobial compounds did not control these bacteria (Zhang et al., 2008). Carminati et al. (2014) after incubation of raw donkey milk samples at 37°C confirmed the ability of the LAB to grow, reaching count values of 8.0 log CFUs/mL. The LAB isolated in this study belonged to the genus *Enterococcus*, *Streptococcus*, and *Pediococcus*, and this was attributed to the higher resistance to lysozyme of coccus-shaped LAB than bacillus-shaped ones. Different strains belonging to the genus *Enterococcus* were isolated from raw donkey milk in Cyprus. They showed safety requirements, with interesting technological and potential probiotic properties that could be useful for the production of biofunctional fermented products (Aspri et al., 2017). Moreover, from donkey milk also mesophilic species of lactobacilli with potential probiotic or biopreservation properties have been previously isolated (Nazzaro et al., 2008; Ashokkumar et al., 2011; Murua et al., 2013).

NUTRITIONAL VALUE AND DIGESTIBILITY OF DONKEY MILK

Lactose is the major component in donkey milk, quantitatively, representing the main energy source, but it also affects bone mineralization as it stimulates, in the neonate, the intestinal absorption of calcium (Schaafsma, 2003). Among minor carbohydrates, with an indirect positive impact on digestibility, donkey milk also contains the same oligosaccharides (3-sialyllactose, 6-sialyllactose,, and disialyl-lacto-*N*-tetraose) found in human milk, but in lower amounts (Monti et al., 2015). The presence of these oligosaccharides, confirming the suitability of donkey milk as infant food, is very important because they have the potential to modulate the growth of intestinal flora, to influence different gastrointestinal and inflammatory processes and to provide protection against bacterial and viral infections (Kunz and Rudolff, 2006).

The consumption of a milk with a high casein content, such as cow milk, induces the formation of a firm curd due to the acid conditions of the stomach, resulting in a delay of protein degradation. Differently, donkey milk, as well as human milk, shows a soft flocculation, rather than an acid coagulation, which is more easily digested and physiologically more suitable for infant nutrition (Barlowska et al., 2011; Uniacke-Lowe et al., 2010). However, protein digestibility is also affected by other factors such as the casein distribution and the size of the casein micelles. The casein-to-whey protein ratio found in donkey milk (1.20–1.00) is more similar to that of human milk (0.64) compared to cow milk (with a ratio of about 4.0), and is believed to play a crucial role in the sensitization to CMPA, reducing its allergenic capacity (Lara-Villoslada et al., 2005). Casein micelle size of donkey milk is large, about 298 nm (Tidona et al., 2014), as it is inversely related to the *k*-casein content (Fox and McSweeney, 1998), and it may be the reason for the high susceptibility to hydrolysis by gastrointestinal enzymes. A first evaluation of donkey milk protein digestibility was performed in vitro, using human gastric and duodenal juices to simulate a human gastrointestinal digestive process (Tidona et al., 2011). Indeed, donkey caseins were easily digested, showing on average only about 7% of native caseins left, after 1 h of digestion. Moreover, the β-lactoglobulins (including types I and II) were highly digestible (about 70% degraded), differing from what was reported for cow and goat milk β-lactoglobulin (Inglistad et al., 2010). The degradation degree was even increased (up to about 90%) in individual milks showing a deficient protein pattern, without β-lactoglobulin type II, and this is nutritionally relevant because human milk is typically devoid of β-lactoglobulins (Tidona et al., 2014). Contrarily, α-lactalbumin has been confirmed as the most-resistant protein (about 95% was undigested), likely reaching the gut almost intact. Another resistant protein was lysozyme (~75% survived from the action of the gastrointestinal enzymes), which is important to inhibit sensitive bacteria in the intestine. Digestibility data regarding some minor whey proteins found in donkey milk (such as lactoferrin, immunoglobulins, serum albumin, and lactoperoxidase) did not produce clear indications due to their low concentration and difficult detectability.

Regarding the lipid fraction, the small globules mostly found in donkey milk have larger amounts of membranes per volume, exhibiting to the digestive enzymes a higher available surface, which is also rich in beneficial components such as PUFAs (Martini et al., 2013). Donkey milk fatty acids are interesting from a nutritional point of view, not only for the composition, mainly unsaturated or short chained, but also for the distribution of fatty acids on the glycerol backbone. In fact, most of the saturated long-chain fatty acids are similar in donkey and human milk, with the sn-2 position mainly occupied by palmitic acid (Claeys et al., 2014). This is an important factor as it determines the lipolysis and thus bioavailability of fatty acids and their possible beneficial effects on health (German and Dillarda, 2006). Finally, despite the higher content of calcium (Ca) and phosphorus (P) found in cow milk in comparison to nonruminant milks, the Ca-to-P ratio on a weight basis of human milk (about 2.1) and donkey (1.5–1.6) is reported to be more favorable for Ca uptake than bovine milk (about 1.2) (Salimei et al., 2004; El-Agamy, 2009).

IMPLICATIONS ON HUMAN HEALTH

The beneficial properties of donkey milk have been known since ancient Egyptian times. Later, Dioscorides (AD 1st century) described the use of donkey milk in human therapy and cosmetics in his *De Materia Medica*, which is considered the milestone of pharmacology (Cosentino et al., 2015a). Beginning in the 19th century, donkey milk began to be regularly used in maternity hospitals to feed orphaned infants, unhealthy children, as well as the ill and elderly (Tesse et al., 2009). Numerous studies reported several health-promoting properties such as antioxidant activity (Perna et al., 2015). In particular, two endogenous peptides from donkey milk proved to exert antioxidant activity, whereas two other endogenous peptides showed angiotensin I-converting enzyme (ACE)-inhibitory activities (Chiozzi et al., 2016). In a study on a rat model, the supplementation of raw donkey's milk for 4 weeks produced, among other effects, a significant increase in antioxidant total thiols and detoxifying enzyme activities [glutathione-S-transferase and nicotinamide adenine dinucleotide hydrogen (NADH) quinone oxidoreductase] in treated animals (Lionetti et al., 2012). Moreover, donkey milk was found rich in some vitamins (A, B_2, C, and E), which also exert antioxidant activities, very important for the slowing down of the aging process and the skin-regeneration effects (Guo et al., 2007). The presence of unsaturated fatty acids such as omega-6, which are highly prized in cosmetics, enables the skin to absorb vitamins to become elastic and prevent certain skin diseases (Iacono et al., 1992). One aspect that was extensively investigated was the antibacterial activity of donkey milk, which successfully proved to inhibit pathogen microorganisms or limit spoilage

(Zhang et al., 2008; Šarić et al., 2012; Fratini et al., 2015). The high lysozyme content is mainly responsible of this activity, but it may also act synergistically with the presence of lactoferrin. In particular, donkey milk digested with human gastrointestinal enzymes showed higher inhibition activity than native milk, attributed to bioactive peptides released during digestion, likely from lactoferrin (Tidona et al., 2011). Additionally, antiviral activity of donkey milk was also reported against echovirus type 5, with the whey protein fraction showing a higher inhibition of virus replication (Brumini et al., 2013).

Administration of donkey milk to aged healthy subjects was able to upregulate the immune response, as shown by the serum cytokine profile (Amati et al., 2010), benefitting human health with special reference to the inflammatory status (Jirillo et al., 2010). Active components, mainly found in the whey protein fraction of donkey milk, were reported to directly suppress lung cancer cell proliferation in vitro, and it may indirectly kill tumors through activation of lymphocytes and macrophages (Mao et al., 2009). These authors concluded that the high content of lysozyme may contribute to the shown antitumor activity.

Despite the low fat content, donkey milk is characterized by a low omega-6 to omega-3 fatty-acid ratio, which gives advantageous values in terms of atherogenic and thrombogenic indices and results, very useful in the prevention of the cardiovascular, autoimmune, and inflammatory diseases (Chiofalo et al., 2001).

PRACTICAL APPLICATIONS FOR DEVELOPMENT OF NOVEL FOODS

The consumption of donkey milk is considered safe and nutritionally valid, and, thanks to the health-promoting properties, it was defined as "pharma food" (Perna et al., 2015). An added value is represented by the suitability of donkey milk as a carrier of probiotic bacteria, which could be useful for the prevention and treatment of antibiotic-associated diarrhea (Coppola et al., 2002; Salminen and Isolauri, 2006; Vincenzetti et al., 2011). Anyway, due to its high lactose content, this milk may be inadequate for people suffering from lactose intolerance. The fermentation of donkey milk using LAB able to hydrolyze lactose, casein, and whey protein with the release of a large number of organic acids, peptides, and amino acids allowed the development of novel foods, useful to meet the needs of consumers with lactose or cow milk protein intolerance. For the success of a food, an essential requirement to satisfy is the sensory evaluation, and donkey milk was positively assessed by panel members, defining it as white, thin, with a slight sweet pleasant taste, milky aroma, sweet flavor, and no persistent aftertaste (Malissiova et al., 2016). This is a prerequisite of milk for drinking purposes, but some technological aspects and suitability for food processing should also be assessed in the derived products, when milk is addressed to manufacturing. Few attempts were performed to produce fermented products of new generation with donkey milk, such as fermented milks, yogurt, or probiotic yogurt (Coppola et al., 2002; Chiavari et al., 2005; Perna et al., 2015). The sensory quality of these products met consumer acceptability, and their functional and probiotic features were confirmed by the survival of LAB starter and probiotic cultures at level above 6 log CFUs/mL at the end of storage (Coppola et al., 2002; Chiavari et al., 2005; Perna et al., 2015). A beverage based on donkey milk supplemented with sunflower oil was also developed. The emulsion of the sunflower oil compensated for the low fat content of donkey milk, improved the viscoelastic properties, and increased some lipid-quality indexes (Tidona et al., 2015). A soft cheese from raw donkey milk was recently obtained by using pure camel chymosin, which was able to clot the casein micelles of donkey milk. The low total solids and casein content, however, make difficult the processing of donkey milk into cheese, obtaining a very low yield (Iannella, 2015). Another interesting application is the employment of donkey milk as an alternative to egg lysozyme, which is used as food additive to prevent blowing defects in semihard and hard cheeses. The addition of donkey milk (from 2% to 8% v/v) increased the relative content of lysozyme in the cow milk to be processed, contributing to the reduction of late blowing defects in cheese, without affecting the sensorial acceptability of the consumers (Cosentino et al., 2015b).

Assessment from an industrial perspective, cow milk is mostly processed in large quantities and is standardized with respect to fat and protein content, whereas equine milk is usually produced on a local scale and, consequently, shows a higher variability in composition due to differences in breed, feeding regime, etc. (Claeys et al., 2014). In the near future, also taking into account the individual productivity of a jenny (Bordonaro et al., 2013), it is assumed a narrow spread of such novel products will appear in the marketplace, but showing substantial room to develop niche market segments. Besides infant diet therapy, the attractive nutritional features shown by donkey milk could also be addressed to other categories (e.g., elderly population) or employed in alternative food formulations.

FINAL REMARKS

Donkey milk can be considered the closest natural milk to human milk, and the results obtained by pediatric scientists seem to confirm the nutritional value of this milk, experienced since ancient times in many countries of the world. The use of donkey milk in diet therapy as an alternative food for infants affected by food allergies is promising but should be supported by more numerous clinical studies in vivo, showing its suitability and safety for human consumption. Considering its unique nutrient profile and economic potential, donkey milk could surely be exploited to fulfill the nutritional requirements of particular consumers and to increase the income of donkey farmers as well. In particular, the consumption of fermented donkey milk might be encouraged to elderly people, thanks to the low caloric intake, good source of bioavailable calcium, and the ability to modulate the aged immune system, including the intestinal mucosal immune response.

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A Novel Donkey Milk–derived Human Milk Fortifier in Feeding Preterm Infants: A Randomized Controlled Trial

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ABSTRACT

Objectives: The purpose of the present randomized controlled clinical trial was to compare the use of donkey milk–derived fortifier (DF) with commercial bovine milk–derived fortifier (BF) in very preterm or very-low-birth-weight newborns, in terms of feeding tolerance.

Methods: This trial included 156 newborns born at <32 weeks of gestational age and/or with a birth weight ≤1500 g. Newborns were randomized 1:1 to receive enteral feeding with either a BF-arm, or a new, DF-arm for 21 days. The fortification protocol was the same for both study arms, and the 2 diets were designed to be isoproteic and isocaloric. Feeding tolerance was assessed by a standardized protocol.

Results: The risk of feeding intolerance tended to be lower in DF-arm than in BF-arm, with a relative risk reduction of 0.63 (95% confidence interval: −0.29, +0.90). The mean number of episodes per newborn of feeding intolerance and feeding interruptions (any duration) were consistently lower in the DF-arm than in the BF-arm. Episodes of bilious gastric residuals and vomiting were significantly lower in the DF-arm. Time needed to reach full enteral feeding ($150\,mL \cdot kg^{-1} \cdot day^{-1}$) and daily weight increase between the first day of exclusive enteral feeding (ie, without administering intravenous fluids) and discharge were similar in the BF- and DF-arms.

Conclusions: These results suggest that DF improve feeding tolerance when compared with standard bovine-derived fortifiers, with a similar auxological outcome.

Key Words: bovine milk, donkey milk, feeding intolerance, human milk fortifier, very low birth weight infants

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What Is Known

- Human milk is the recommended food for preterm newborns; however, it has to be fortified.
- At present, the most common fortifiers are bovine milk derived. The optimal composition of human milk fortifiers is still debated.

What Is New

- A new donkey milk–derived human milk fortifier is suitable for feeding preterm and very-low-birth-weight newborns.
- The donkey milk–derived fortifier, compared to a bovine counterpart in an isocaloric and isoproteic diet, seems to improve feeding tolerance, with a similar auxological outcome.

Very preterm (gestational age <32 weeks) and very-low-birth-weight (VLBW, ie, <1500 g) newborns currently represent the majority of patients admitted to neonatal intensive care units (NICU) (1). Improvements in perinatal care have led to an increased survival rate in these newborns, which has offered new insights into their outcome and their health status in adulthood.

Nutrition is fundamental to neonatal survival and short-term outcomes, but it also has long-term consequences on quality of life in very preterm and VLBW newborns. Indeed, these newborns require adequate qualitative and quantitative nutrition, particularly in terms of protein intake, the lack of which is the main cause of postnatal growth deficits (2). Human milk is the recommended food

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E.B., L.C., G.E.M., and A.C. have competing interest since they are the inventors of a patent on the fortifier derived from donkey milk described in the paper (Italian Patent no. n.0001421271 and international patent application no. WO2015056166 (A1)-20,150,423). The remaining authors report no conflicts of interest.

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for all neonates (3,4), but as breast milk alone does not meet the nutritional requirements of preterm newborns (5,6), it is supplemented with additional nutrients (7,8).

Fortification of human milk still represents a significant challenge (9–11), as concerns have been raised regarding fortification strategies and the composition of fortifiers. Individualized fortification is the current recommended strategy (12,13), and fortifiers must be composed of simple, high-quality, well-tolerated nutritional supplements. Recently, human milk–based fortifiers have been proposed, but their utilization is limited by high costs and ethical issues. Moreover, there is no strong evidence that human milk–based fortifiers in otherwise exclusively human milk–fed preterm infants affect important outcomes. (14).

Based on its physiochemical properties, milk from monogastric animals has been suggested to be more suitable than bovine milk for human nutrition (15). Donkey milk showed biological effects comparable with those elicited by human milk (16,17). Our hypothesis is that feeding very preterm and VLBW newborns with human milk supplemented with donkey milk–derived fortifiers (DFs) will improve feeding tolerance. Thus, the present trial compared the use of DF and commercial bovine milk–derived fortifier (BF) in very preterm and VLBW newborns, in terms of feeding tolerance and short-term auxological outcomes.

METHODS

This study was performed in the NICU of Turin University. It was approved by Ethics Committee (AN: 0025847, 27/05/2014) and registered (*http://www.isrctn.com/ISRCTN70022881*, ISRCTN70022881) after the trial starting date. The study protocol was evaluated by JPGN Editorial Office. Recruitment period was 27/11/2014 to 22/12/2016. Written informed consent was obtained from the parents of all included newborns before enrollment.

Study Population

The inclusion criteria were gestational age <32 weeks and/or birth weight ≤ 1500 g, exclusive feeding with human milk (own mother's milk or donor milk), and enteral feeding $\geq 80\,\text{mL}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ within the first 4 weeks of life. Newborns with severe gastrointestinal pathologies (necrotizing enterocolitis, colostomy, intestinal obstruction, symptoms of peritonitis, presence of blood in the feces), chromosomal abnormalities or major malformations, hereditary metabolic diseases, intravascular disseminated coagulopathy, shock, patent ductus arteriosus (PDA) requiring medical care or surgery at the time of randomization, and severe renal failure (serum creatinine >2 mg/dL) were excluded.

Study Design

Eligible newborns were randomly allocated 1:1 into 2 arms in accordance with a list generated by a data step written in SAS (18) language: the BF-arm and the DF-arm. In the BF-arm, a bovine milk–derived commercial multicomponent fortifier (FM85, Nestlé) and a bovine milk–derived protein concentrate (Protifar, Nutricia, Utrecht, The Netherlands) were used. In the DF-arm a donkey milk–derived multicomponent fortifier and donkey milk–derived protein concentrate were used (FortiLat, Torino, Italy). The DF is not commercially available and was produced according to current EU legislation on food for special medical purposes.

All newborns received enteral feeding according to a regimen of *adjustable fortification*, based on blood urea nitrogen determination, for 21 days (19,20). The intervention started when the infants were able to tolerate a volume of $\geq 80\,\text{mL}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ (randomization time) and, according to study protocol, was planned to last 21 days; the intervention was suspended at discharge from the hospital for any reason (transfer, death, discharge home).

Please refer to our previous article (21) for a detailed description of the methodology used in the study. Because the protein concentration and energy content of bovine milk–derived products differ from those of donkey milk–derived products, the amounts of powder required to obtain the same level of fortification were different. Moreover, because the same nurses were in charge of both the preparation and administration of meals and the evaluation of feeding tolerance, this study must be regarded as an open-label trial. Increases in the quantity of milk given during enteral feeding were strictly regulated according to the feeding protocol adopted in the NICU, based on the evaluation of signs of feeding intolerance. Data on necrotizing enterocolitis that occurred after randomization, PDA, sepsis, mortality, hospital stay duration, intraventricular hemorrhage, and retinopathy of prematurity (defined according to the Vermont Oxford Network) (22) were collected from hospital records.

Babies were discharged from the hospital when they met all following criteria: satisfactory weight gain while receiving full oral feeding, maintenance of adequate thermal stability, and resolution of acute medical conditions.

Outcome Measures of Feeding Tolerance

Primary Endpoint

Primary endpoint includes death, necrotizing enterocolitis, or at least 1 episode of feeding intolerance, defined as interruption of enteral feeding for at least 8 consecutive hours during the observation period.

Secondary Endpoints

Secondary endpoints include number of episodes of feeding intolerance, feeding interruption (any duration), bilious gastric residuals, vomiting, and total hours of enteral feeding interruption.

Time required to reach full enteral feeding ($150\,\text{mL}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) and daily weight gain (weight-Δ standard deviation score (ΔSDS)/days) from the first day of exclusive enteral feeding (without administering intravenous fluids) until discharge were also evaluated.

Study Size

The evaluation of the previous year's hospital records, carried out before the start of the present study, revealed that approximately 45% of very preterm or VLBW newborns admitted to the NICU had at least 1 episode of feeding intolerance (primary endpoint). A 25% reduction in the frequency of the primary endpoint was regarded as the minimum clinically important difference; under these assumptions, 62 newborns per arm had to be recruited to ensure an 80% study power, given a risk of type I error at the usual level of 5%. However, the occurrence of the primary endpoint was much lower than that assumed in the protocol, and no adverse effect of FortiLat was observed. Because the occurrence of primary endpoint in our study population resulted to be much lower than that assumed in the protocol, and no adverse effect was observed, when the planned study size was achieved, it was decided to continue the enrollment until the stock of FortiLat ran out. For this reason, we present information on the planned study with the initial 62 newborns per arm, and the extended study, with the additional recruitment. A further randomization list was generated for the extension of the study.

Statistical Analysis

The analysis was performed on 2 populations:

- All randomized subjects (ARS) population, which included all randomized newborns.
- Per-protocol (PP) population, which included only newborns observed for 21 days in our hospital, and that actually received donkey milk or bovine fortifier according to the protocol, excluding, consequently, the babies transferred to other hospitals or discharged home before 21 days of observation.

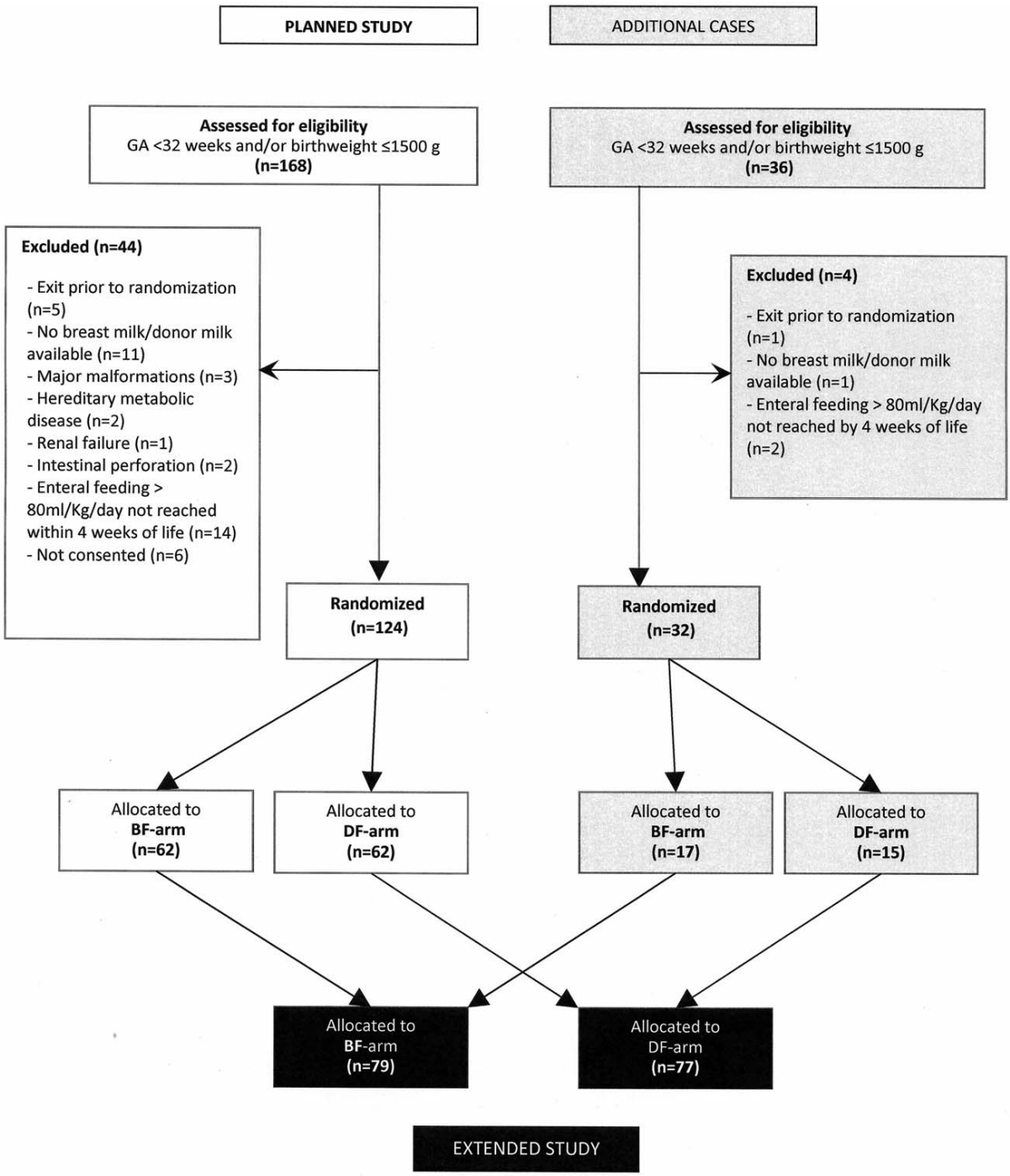

FIGURE 1. Diagram of the enrollment, randomization, and study allocation. BF = bovine milk–derived fortifier; DF = donkey milk–derived fortifier.

The primary endpoints were evaluated both in the ARS and in the PP population. In the ARS population, the analysis (primary analysis) was performed in accordance with the intention-to-treat approach: failure included all the conditions that cannot be defined success, that is, occurrence of necrotizing enterocolitis, at least 1 episode of feeding intolerance, death, or transfer to another hospital before day 21 of observation. Subjects in the ARS population who were discharged home before the 21st day were considered successes, under the assumption that they maintained good tolerance at home. In the PP population, from which subjects transferred to another hospital or discharged before the 21st day are excluded, the occurrence of death, necrotizing enterocolitis, and at least 1 episode of feeding intolerance were regarded as failure.

The difference in the outcome between the 2 study arms was tested with the Fisher exact test. The risk of recurrent episodes of feeding intolerance (interruption of enteral feeding for at least 8 hours) in the 2 arms was estimated on the ARS population with the Andersen and Gill Cox's model for recurrent processes (23).

The analysis of secondary endpoints, because time-dependent, was carried out on PP population, resorting to generalized linear models (24): the number of episodes of feeding intolerance, feeding interruptions (any duration), bilious gastric residuals, and vomiting occurred during the observation were modeled as a Poisson variable; total hours of enteral feeding interruption were modeled, after log-transformation, as normal variables. Median time required to reach full enteral feeding was estimated on the ARS population according to Kaplan and Meyer (25). Body weight was expressed as SDS, with respect to Italian Neonatal Study (INeS) charts (26). To evaluate differences in growth between the 2 arms, weight gain was expressed as weight-ΔSDS/days, that is, the mean daily weight-SDS variation between SDS on the first day of exclusive enteral feeding and discharge. In this analysis, only babies discharged home were considered.

TABLE 1. Maternal and neonatal characteristics, clinical condition at randomization, and clinical outcome and morbidities during the observation period

		Planned study		Extended study	
		BF-arm (n = 62)	DF-arm (n = 62)	BF-arm (n = 79)	DF-arm (n = 77)
Maternal characteristics					
Pregravidic BMI, kg/m^2	Mean (SD)	23.7 (4.53)	23.6 (5.70)	23.4 (4.47)	24.0 (5.96)
Weight gain in pregnancy, kg	Mean (SD)	9.0 (6.02)	8.2 (6.14)	8.7 (6.00)	8.8 (5.94)
Age, y	Median (IQR)	33.5 (30–38)	34.5 (30–39)	34 (30–38)	34 (30–39)
Chronic diabetes	n (%)	0 (0.0)	1 (1.6)	0 (0.0)	1 (1.3)
Chronic hypertension	n (%)	3 (4.8)	2 (3.2)	3 (3.8)	3 (3.9)
Gestational diabetes	n (%)	10 (16.1)	11 (17.7)	11 (13.9)	14 (18.2)
Gestational hypertension	n (%)	18 (29.0)	11 (18.0)	22 (27.8)	12 (15.8)
Caesarean delivery	n (%)	50 (80.6)	46 (74.2)	58 (73.4)	60 (77.9)
Prelabor rupture of membranes	n (%)	17 (27.4)	15 (24.2)	23 (29.1)	17 (22.1)
Assisted reproductive technology	n (%)	15 (24.2)	12 (19.4)	19 (24.1)	13 (16.9)
Neonatal characteristics					
Boys	n (%)	29 (46.8)	31 (50.0)	36 (45.6)	37 (48.1)
Singletons	n (%)	35 (56.5)	40 (64.5)	47 (59.5)	46 (59.7)
Firstborn	n (%)	40 (64.5)	39 (62.9)	51 (64.6)	50 (64.9)
Gestational age <32 wk*	n (%)	50 (80.6)	48 (77.4)	64 (81.0)	55 (71.4)
VLBW (birth weight ≤1500 g)†	n (%)	57 (91.9)	53 (85.5)	70 (88.6)	65 (84.4)
Small for gestational age‡	n (%)	16 (25.8)	20 (32.3)	19 (24.4)	27 (35.1)
Weight, g	Mean (SD)	1166 (297.3)	1196 (315.7)	1161 (310.3)	1214 (311.5)
Weight (SDS)	Mean (SD)	−0.36 (1.122)	−0.64 (1.165)	−0.35 (1.120)	−0.74 (1.162)
RDS	n (%)	53 (85.5)	54 (87.1)	69 (87.3)	67 (87.0)
Age at randomization, days	Median (IQR)	9.0 (6–17)	8.5 (5–14)	9.0 (6–17)	8.0 (5–14)
Age at start of intervention, days	Median (IQR)	11.5 (8–17)	10.5 (7–17)	12.0 (8–18)	11.0 (7–17)
Intraventricular hemorrhage	n (%)	5 (8.1)	2 (3.2)	8 (10.1)	3 (3.9)
Recovered patent ductus arteriosus	n (%)	20 (29.4)	11 (16.2)	26 (38.5)	11 (16.2)
Clinical outcome and morbidities					
Length of hospital stay§	Median (IQR)	45 (32–63)	39.5 (29.5–63)	45.5 (32–63)	38 (28–56)
Transferred to other hospital	n (%)	6 (9.7)	5 (8.1)	7 (8.9)	5 (6.5)
Dead before discharge	n (%)	1 (1.6)	1 (1.6)	1 (1.3)	1 (1.3)
Steroids therapy	n (%)	1 (1.6)	1 (1.6)	1 (1.3)	1 (1.3)
Early sepsis	n (%)	3 (4.8)	1 (1.6)	4 (5.1)	1 (1.3)
Late sepsis	n (%)	4 (6.5)	3 (4.8)	5 (6.3)	3 (3.9)
Necrotizing enterocolitis	n (%)	1 (1.6)	1 (1.6)	1 (1.3)	1 (1.3)

BF = bovine milk–derived fortifier; BMI = body mass index; DF = donkey milk–derived fortifier; IQR = interquartile range; RDS = respiratory distress syndrome; SD = standard deviation; SDS = standard deviation score; VLBW = very-low-birth weight.

*Regardless of birth weight.

†Regardless of gestational age at birth.

‡Birth weight below the 10th centile of Italian Neonatal Study (INeS) charts [26].

§Computed on babies discharged to home.

SAS software was used to process data and fit statistical models (18).

RESULTS

The ARS population consisted of 124 newborns enrolled in the planned study (BF-arm: n = 62; DF-arm: n = 62). During the extended study 32 more newborns were enrolled, for a total of 156 babies (BF-arm: n = 79, DF-arm: n = 77) (Fig. 1). The PP population (patients that completed 21 days of observation) was made up of 89 newborns (BF-arm: 44, DF-arm: 45) in the planned study and 111 (BF-arm: 57, DF-arm: 54) in the extended study. No babies switched from one arm to the other.

Table 1 shows the characteristics of mothers and neonates included in the planned and extended study before enrollment and clinical outcome and morbidities that occurred during the observation period. In the table, PDA refers to a condition from which the newborn recovered before randomization. The median time lag between the random assignment of subjects to either arms and the actual start of the intervention did not exceed 3 days. One baby per arm died following necrotizing enterocolitis.

Primary Endpoint

The number of failures and successes observed in the 2 arms for the planned and the extended study is reported in Supplementary Table 1 (top) (Supplemental Digital Content, *http://links.lww.com/MPG/B499*). Risk of failure in the planned study tended to be lower in the DF- than in the BF-arm, with a relative risk reduction of 0.40 (95% confidence interval [CI]: −0.27, +0.72; Fisher exact test: $P = 0.256$) in the ARS and 0.63 (95% CI: −0.29, +0.90; $P = 0.118$) in the PP population (Fig. 2, left). Results were similar in the extended study, with relative risk reductions of 0.46 (95% CI: −0.09, +0.73; $P = 0.100$) in the ARS and 0.58 (95% CI: −0.27, +0.86; $P = 0.153$) in the PP population (Fig. 2, right).

Secondary Endpoints

The number of episodes of feeding intolerance, feeding interruptions (any duration), bilious gastric residuals, and vomiting for the planned and extended study observed in the PP population is reported in Supplementary Table 1 (bottom) (Supplemental Digital Content, *http://links.lww.com/MPG/B499*). During the observation period, the mean number of episodes per newborn of these secondary endpoints was consistently lower in the DF- than in the BF-arm. Indeed, the difference between the BF- and the DF-arm ranged from 0.09 to 0.31 in the planned study (Fig. 3, left), and from 0.15 to 0.35 in the extended study (Fig. 3, right). In the extended study, the difference between the arms was statistically significant as regards the number of episodes of bilious gastric residuals ($P = 0.009$) and vomiting ($P = 0.041$).

The hazard ratio of recurrent feeding intolerance episodes (DF-arm vs BF-arm), estimated in the ARS population using Anderson and Gill Cox's model for recurrent processes, was 0.53 (95% CI: 0.20, 1.44; $P = 0.215$) in the planned study, and 0.40 (95% CI: 0.17, 0.95; $P = 0.038$) in the extended study.

The median time to achieve full enteral feeding in the BF- and DF-arms was 19 days (95% CI: 15, 23), both in the planned and in the extended study. The total number of hours of feeding interruptions did not differ significantly between the 2 arms, neither in the planned study (BF-arm: 1.28; 95% CI: 0.50, 2.49 and DF-arm: 0.68; 95% CI: 0.10, 1.55; $P = 0.304$) nor in the extended study

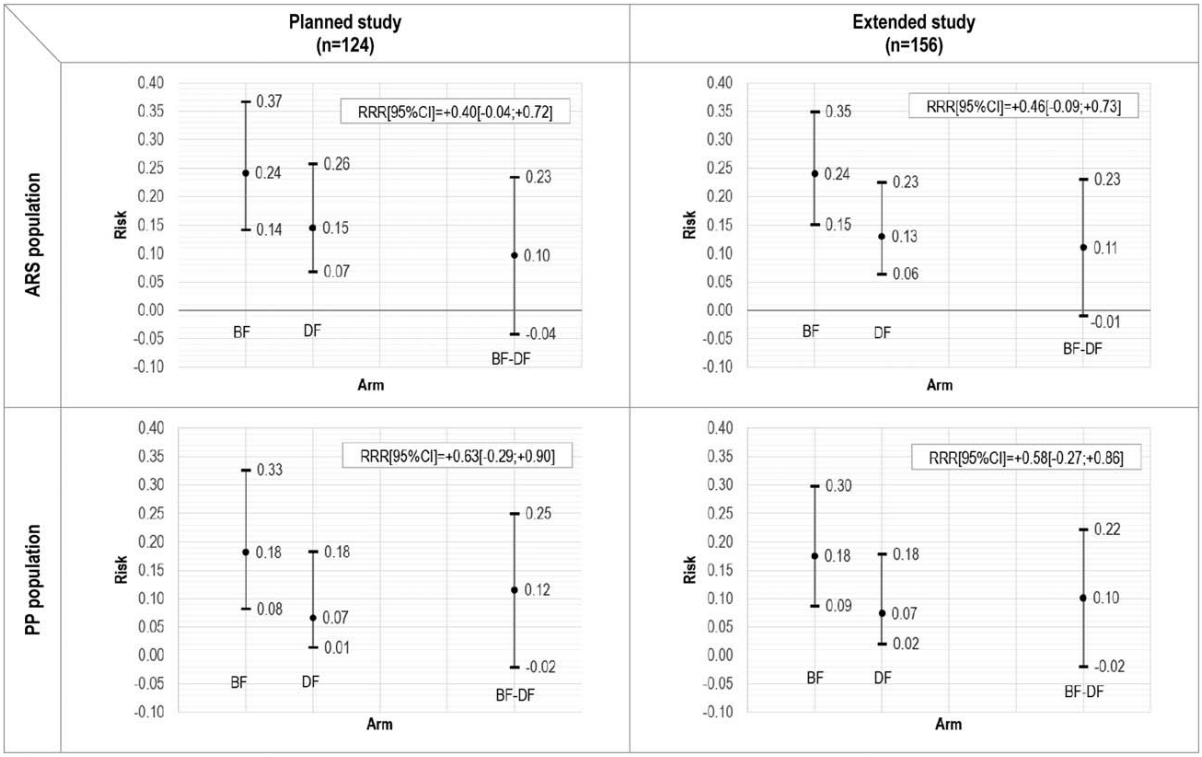

FIGURE 2. Primary endpoint: risk of failure in the 2 arms and relative risk reduction (RRR), and 95% confidence intervals. ARS = all randomized subjects; PP = per-protocol.

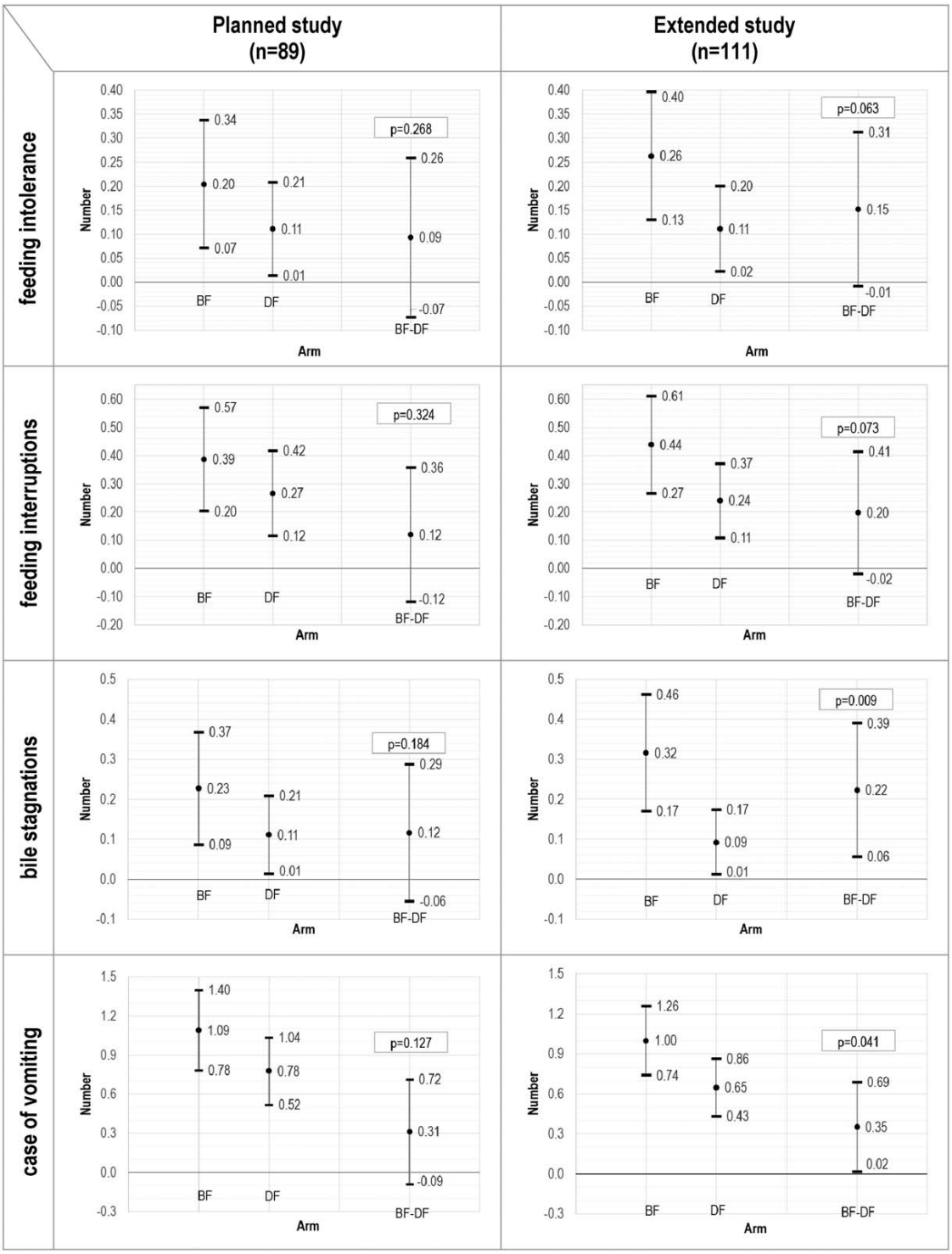

FIGURE 3. Secondary endpoints (on PP population): means, mean differences, and 95% confidence intervals.

(BF-arm: 1.15; 95% CI: 0.49, 2.12 and DF-arm: 0.66; 95% CI: 0.14, 1.44; $P = 0.340$).

Mean daily weight increase between the first day of exclusive enteral feeding and discharge (expressed as ΔSDS/day) did not differ between the BF-arm (−0.013; 95% CI: −0.018, −0.009) and the DF-arm (−0.012; 95% CI: −0.016, −0.008) in the planned study. Similar results were observed in the extended study (BF-arm: −0.012; 95% CI: −0.016, −0.008; DF-arm: −0.013; 95% CI: −0.018, −0.008).

DISCUSSION

The aim of the study was to assess the effects of a donkey milk–derived human milk fortifier on feeding tolerance among very preterm (gestational age <32 weeks) and VLBW (≤1500 g) newborns. To the best of our knowledge, our trial is the first to investigate the use of a DF for the nutrition of very preterm and VLBW newborns. All newborns (both the BF-arm and the DF-arm) received human milk exclusively (raw own mother's milk or pasteurized donor milk), without any preterm bovine formula supplementation. In contrast, Sullivan et al (27) included subjects receiving preterm formula in the group supplemented with the bovine fortifier in their comparison of a human milk–based and bovine milk–based fortifier, which represents a confounding variable.

In our study, we observed a lower number of failures (necrotizing enterocolitis, at least 1 episode of feeding intolerance, or death) and a lower hazard of feeding intolerance episodes in the DF-arm, both in the planned and in the extended study, in ARS and in PP population. The mean number of episodes per newborn of feeding intolerance, feeding interruptions (any duration), bilious gastric residuals, and vomiting during the observation period was consistently lower in DF-arm, both in the planned study and in the extended study. Overall, these results suggest the favorable effect of the donkey milk fortifier on feeding tolerance, which could not be demonstrated due to the unexpected lack of power of our study. Actually, our study was planned under the assumption that the occurrence of failures (necrotizing enterocolitis, at least 1 episode of feeding intolerance, or death) in the control arm (BF-arm) was 45%, whereas during the trial it was only 24%. This could be due to the so-called *Hawthorne effect* (28,29), that is, to the fact that the behavior of clinical staff may be affected and improved when a trial is conducted in a clinical setting. Because of the lower occurrence of failures, the statistical power to detect a decrease from 24% to 11% (ie, the same relative decrease in failure occurrence assumed in the protocol), was only 38% (about half of the prefixed 80%) in the planned study and 48% in the extended study. Under these conditions, it would have been necessary to enroll 148 subjects per arm to achieve an 80% power.

Overall, a better tolerance of DFs emerged. We speculate that the quality of donkey milk protein could be responsible of this result, the 2 diets being isoproteic and isocaloric. Weight gain was similar in BF- and DF-arms, suggesting that differences in tolerance do not affect short-term growth, at least under the conditions on which this trial was carried out, where a parenteral intake was provided in case of episodes of enteral feeding intolerance and suspension. For this reason, a similar total nutritional intake was provided in all subjects. At present, commercially available fortifiers are bovine milk derived, with a protein composition that is very different from that of human milk. Bovine milk whey proteins contained in the fortifier used in this study strongly differ from human milk counterparts in term of relative abundance and primary structure (30). The intake of bovine milk protein in the first months of life has raised concerns because of its association with allergies (31). Furthermore, bovine milk has been reported as a possible trigger of intestinal inflammation in preterm neonates (32,33). Previously, we found that the protein and lipid fractions in donkey milk are similar to those in human milk (30,34). We also observed that donkey milk was well tolerated in a group of children with highly problematic cow's milk allergies (35). Moreover, it has recently been demonstrated in murine models that a supplementation of the basal diet with donkey milk decreases the accumulation of body lipids and affects glucose and lipid metabolism in a manner more similar to human than to bovine milk. These biological effects are comparable to those elicited by human milk (16,17). Based on the above-mentioned studies and the results obtained in the present trial, it can be hypothesized that donkey milk is more suitable than bovine milk as an ingredient in human milk fortifier for very preterm and VLBW newborns.

For a more comprehensive evaluation of the results, we should consider that the 2 arms slightly differed: a higher number of newborns developed PDA in the BF-arm before randomization (and PDA at the time of randomization was an exclusion criterion), small for gestational age (SGA) newborns were more frequent in the DF-arm, whereas VLBW newborns were more frequent in the BF-arm.

The presence of symptomatic PDA may theoretically impair feeding tolerance because of the impact on blood flow to vital organs (36,37), but at time of randomization this condition had been resolved. SGA newborns are at higher risk for intestinal disturbances, ranging from temporary enteral feeding intolerance to necrotizing enterocolitis. In our study, the best tolerance was observed in the DF-arm, in which SGA subjects, who were at major risk of feeding difficulties, were more numerous.

A limitation of this trial is that it was designed as an open-label randomized clinical trial, because the nurses in charge of the preparation of meals were also in charge of evaluating signs of feeding tolerance. The nurses, however, involved in the trial should stick to a strict protocol to reduce their discretion in the evaluation of signs of feeding intolerance.

To conclude, the new DF was well tolerated in our population. The results of this trial may constitute a sound basis on which to plan a further trial with enough power to confirm the higher tolerability of the DF and open new perspectives for the production of human milk fortifiers other than those derived from bovine milk.

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TOLERABILITY OF DONKEYS' MILK IN 92 HIGHLY-PROBLEMATIC COWS' MILK ALLERGIC CHILDREN

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Objective: Not exclusively breastfed children with cows' milk allergy (CMA) require a formula or other alternative food, but past and present guidelines differ concerning the best choice. Our aim was to investigate the clinical tolerability, palatability and nutritional adequacy of donkeys' milk (DM) in children with proven CMA. It was important to identify a CM replacement for these children, highly problematic from the feeding standpoint, in spite of their age. Study design: A prospective study was conducted on 92 children with CMA, diagnosed through a CM elimination diet, followed by double-blind, placebo-controlled food challenge (DBPCFC) unless contraindicated. Maternal milk was unavailable and current CM substitutes could not be used. Moreover, 89% were affected by multiple FA, and subjected to very restricted diets. Within 3 months after the last CM challenge, DBPCFC for DM was performed. CM or DM skin prick test and sIgE determination preceded the CM or DM challenge, respectively. Native electrophoresis and immunoblotting were used to identify CM and DM cross-reactive proteins. Z-scores of weight and length/stature for age were calculated at DM food challenge (T_0) and during DM assumption. Results: 83 children (90.2%) liked and tolerated DM, at challenge and during follow-up, with increased Z-score for weight and length/stature and improved nutritional parameters. Bovine beta-lactoglobulin was identified as the cross-reacting protein among the DM allergic patients. Conclusions: DM was found to be a valid alternative foodstuff, in terms of clinical tolerability, palatability and nutritional adequacy, in subjects with CMA who were highly problematic from the feeding standpoint.

Treatment of cows'milk allergy (CMA) hinges upon the complete elimination of cows'milk proteins (CMP) from the diet. In non-breastfed infants, in children under two-three years of age, and in children above 2-3 years with CMA associated with allergies to other staple foods, it is mandatory to find a substitute formula or other alternative food [1].

The selection of a formula depends on the allergy syndrome to be treated. Current cows' milk (CM) substitutes are soy-protein-based formulas (SF), extensively hydrolized formulas of either CM proteins (eHF) or rice proteins (RHF), and amino-acid formulas (AAF). There is no agreement concerning the best choice, in recent [1-3] nor previous guidelines [4,5]. All these substitutes have an acceptable nutritional value, although the long-term effects in infants have yet to be studied [6,7]. Moreover, many questions remain open concerning the high concentrations in SF of phytate, aluminium and phyto-estrogens (isoflavones). The chief limitation upon the use of eHF, RHF and AAF consists of their extremely unpleasant taste [8-10]. For these reasons, alternative milks from other mammalian species have been considered [9, 11-19]. Equine milks (mares' and donkeys' milk) were the object of clinical studies on very small series [11,13,14], and recently on larger series, in vivo [18,19] or both in vivo and in vitro [9], with high rates of tolerability. This prospectic study investigated the efficacy of donkeys' milk (DM) in terms of clinical tolerability, palatability and nutritional adequacy, in

Key Words: cows' milk allergy, alternative milk substitutes, donkeys' milk

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an even larger series of children with proven CMA, highly problematic from the feeding standpoint, and identified any CM and DM cross-reactive proteins.

MATERIALS AND METHODS

Subjects and study design

Between 1 February 2004 and 1 July 2009, 92 children were recruited (55 boys; age-range 7.5-121.5 months, median 19.5, mean 27.3) with proven CMA, for whom maternal milk was unavailable and current CM substitutes could not be used.

Table I shows age and symptoms at first observation. At recruitment, 82/92 children (89%) suffered from allergy to other foods (mainly eggs, wheat, and fish).

Unless contraindicated [1], the diagnosis of CMA was made on the basis of a CM elimination diet (2-4 weeks), followed by double-blind, placebo-controlled food challenge (DBPCFC). Before food challenge (FC), skin prick tests (SPT) were done for CM, and sIgE for cows' milk proteins (CMP) were determined. At recruitment, the parents of 8 children (mean age 28 months) preferred to avoid current CM substitutes, but rather to try DM directly; the parents of a further 3 children (mean age 16 months) who were already taking SF wished to try DM. Of the remaining 81/92 children, 53 already at first observation presented GI symptoms such that it was inappropriate to use SF [1,3], whereas the other 28 (30.4%) presented an allergy to SF between first observation and recruitment, confirmed by positive DBPCFC. SF could therefore not be used for any of these 81 subjects, and it was also impossible to use either eHF, RHF or AAF: 7/81 children did not tolerate eHF and refused to take RHF or AAF, because of their unpleasant taste; the remaining 74/81 categorically and systematically refused to take either eHF/RHF or AAF, some immediately at the first attempts, others after taking those formulas for variable periods of time (12 months on average). It was thus proposed to the parents of these 81 children that they participate in the study using DM to replace CM.

Ethical approval was obtained from the local Review Board, and informed parental consent was given. DM was obtained from a certified organic farm where donkeys are raised outdoors and fed exclusively with organic foodstuffs.

Skin tests

CM or DM SPT were performed before CM or DM challenge, respectively with fresh CM, or with fresh DM, as described elsewhere [20].

Determination of specific IgE

Serum levels of CMP-sIgE and DM proteins (DMP)-sIgE were determined by the automated Pharmacia CAP system FEIA (Phadia & Upjohn Diagnostics, Sweden) on blood samples taken during the SPT.

Food challenge

Both CM and DM food challenge were performed following Monti et al. [9]. The FC for DM was performed during a period of 0.5-3 months after the last FC for CM, using fresh DM after heating to 70° for 2 min.

Follow-up

The study design included clinical and auxological follow-up, at DM food challenge (T_0), and after 1 month (T_1), 2-3 (T_2), 4-6 (T_3), 7-12 (T_4), 13-18 (T_5), 19-24 (T_6), 25-36 (T_7), and 37-48 months (T_8) of DM consumption. The last evaluation was defined as T_{end} and corresponded to the moment when the child stopped ingesting DM. Auxological evaluation was performed following Monti et al. [9]. The child's diet was appropriately balanced depending on requirements by age by a dietician. The parents kept a food diary, with particular reference to daily DM consumption. Resolution of the CMA was assessed periodically during follow-up. For the 92 children, at T_0 and after 6-12 months (T_c) of DM ingestion, parental consent was requested to evaluate the following nutritional parameters: blood count, iron status (serum iron, transferrin, saturated transferrin, ferritin), calcium-phosphorus balance (calcium, phosphorus, alkaline phosphatase, vitamin D), protein balance (serum prealbumin, IGF-1), lipid balance (total cholesterol and HDL, triglycerides, apolipoproteins A1 and B, and their ratio).

Statistical analysis

Z-scores of weight (WA) and length/stature (LA) for age were calculated from the formula $Z = x - |X| / |SD|$, taking the Gardner and Pearson growth curves as reference for children up to 24 months and the Tanner curves after 2 years of age, to evaluate changes in anthropometric parameters independently of age. The differences in Z-score between T_0 and T_1 and between T_0 and T_{end} were evaluated by the t-test for paired data. Z-score differences between check-ups were analyzed with the ANOVA test for repeated measures. A post hoc analysis was performed through the Bonferroni multiple comparisons test to establish the significance of differences between check-ups. The difference in blood-chemistry parameters between T_0 and T_c was evaluated through the t-test for paired data, or the Wilcoxon test for paired data, as appropriate. Significance was set at $p < 0.05$. Statistical analysis was performed with the Stat 5.5 software (StatSoft, Inc).

Native Electrophoresis and Electroblotting

For native electrophoresis, skimmed DM was mixed with boric acid-borax buffer 0.2M, pH 8.4, "Sample buffer" (0.5M Tris-HCl pH 6.8, 0.5% (w/v) Bromophenol blue and glycerol) and water (1:1:1) and brought up to 200 µl. Each gel was loaded with 150µg of total protein, following Bolt et al. [21]

Immunostaining

Immunolabeling was performed according to Natale et al. [22] with serum from 17 patients: of these, 12 only had FC positive to CM, 5 also had FC positive to DM.

Mass spectrometry

DMP were identified by MALDI-TOF (Ultraflex II TOF/TOF, Bruker) after reduction and alkylation according to Fortunato et al. [23]. The proteins were then digested in gel with trypsin, following Bertino et al [24].

RESULTS

Table I reports the outcome of SPT for CM and sIgE

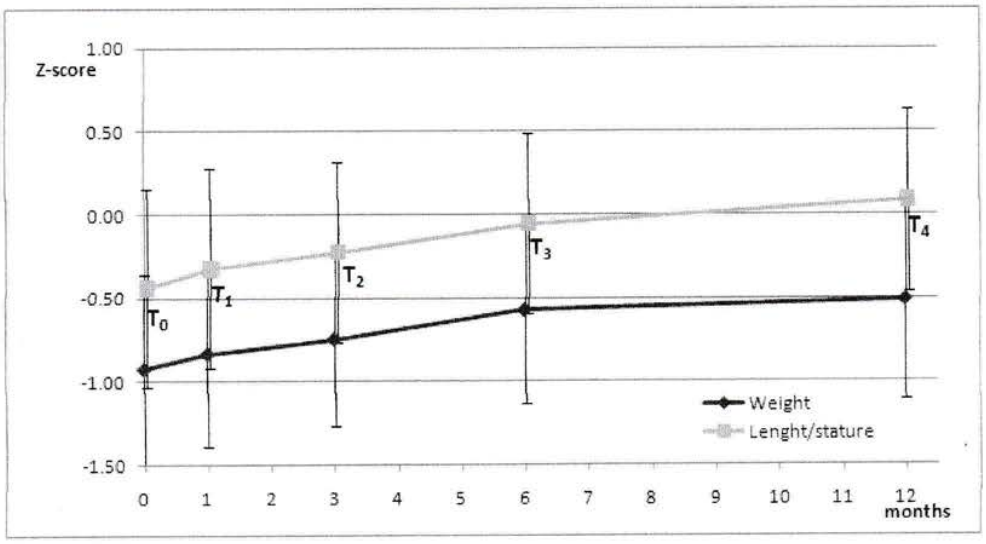

Fig. 1. *Weight and length/stature Z-score variations in the first year of uninterrupted DM feeding.*

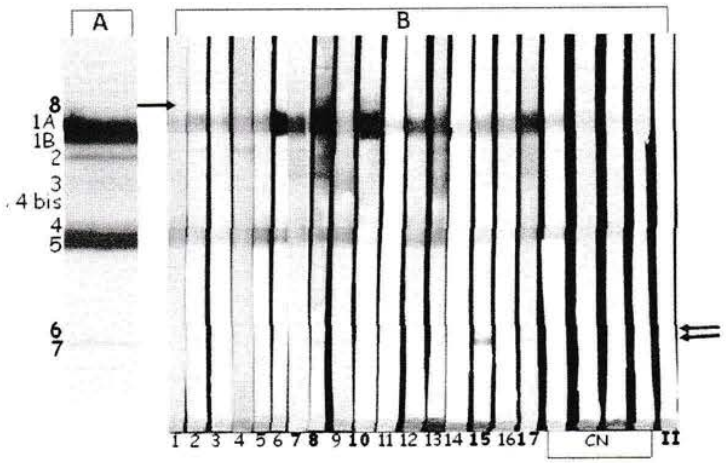

Fig. 2. *Native Electrophoresis and Immunoblotting. A = Native blotting on NC membrane of fresh donkeys' milk proteins stained by Sypro Ruby blot staining; B = Native Immunoblotting using as primary antibody sera from 17 different patients. In bold type, patients with oral challenge positive for both donkeys' and cows' milks (patients 7, 8, 10, 15 and 17); the remaining 12 patients were only allergic to cows' milk proteins. Secondary antibody: alkaline phosphatase-conjugated goat anti-human-IgE. CN = negative controls, sera of four non-allergic subjects; II = secondary Ab control, immunoblotting without primary Ab.*

levels for CMP, as well as age and clinical symptoms at the last CM food challenge, prior to enrolment. As patients with a recent history of anaphylaxis, or in whom a severe allergic reaction had occurred immediately after CM exposure and with positive CM IgE tests should not be challenged [1], CMA was confirmed with DBPCFC in 79/92 children. Sixty-nine children (75%), with SPT and/or sIgE positive for CMP, had an IgE-mediated CMA.

83/92 children (90.2%) both liked and tolerated DM at the challenge and for the entire duration of follow-up. DM was tolerated by 20/23 (87%) children with non-IgE-mediated CMA and by 63/69 children (91.3%) with IgE-mediated CMA. In particular, all 11 children with prior anaphylaxis due to CMA tolerated DM, as did both

Table I. *Clinical characteristics of each patient*

	FIRST OBSERVATION		PbP CMP	CMP sIgE (KU/L)			CMP CHALLENGE		PbP DM	DM sIgE	DM CHALLENGE	
N	AGE (months)	SYMPTOMS	(ø mm)	CAS	ALA	BLG	Age (months)	Symptoms	(ø mm)	(KU/L)	Age (months)	Symptoms
1	37	AD, DPE	0 (NEG)	0,09	0,09	0,09	40	G, AP (e)	0 (NEG)	0,09	43	-
2	9	AD, FTT	15 (POS)	11,80	10,40	4,32	19	AD (l)	0 (NEG)	1,41	20	-
3	38	CD, FTT	2 (NEG)	0,31	2,60	0,19	38	D (e)	0 (NEG)	0,37	40	-
4	54	**AN (CMA), AD, AP**	8 (POS)	0,69	24,64	0,01	-	not performed	0 (NEG)	0,01	58	-
5	12	U A, FTT	6 (POS)	0,46	0,04	0,58	13	U A (l)	0 (NEG)	0,01	14	-
6	29	DPE	0 (NEG)	0,02	0,01	0,03	30	D (l)	0 (NEG)	0,01	33	-
7	8	AD, severe IC, CD	8 (POS)	1,41	0,34	0,67	9	U, D, NS (l)	0 (NEG)	0,09	10	-
8	6	AD, FTT	8 (POS)	2,94	0,00	0,08	10	U (l)	0 (NEG)	0,19	13	-
9	120	AD, GERD, AP, FTT	3 (POS)	0,09	0,09	0,09	121	AP (e), WL (l)	0 (NEG)	0,09	121,5	AP (l)
10	2	DPP	11 (POS)	4,76	3,45	1,72	34	D, AP (l)	3 (POS)	0,09	36	NS, D (l)
11	4	AD, DPP	0 (NEG)	0,09	0,09	0,09	15	AP (e), AD (l)	0 (NEG)	0,12	16	-
12	50	AD, U, A, DPP*	0 (NEG)	0,06	0,20	0,31	50,5	AP (e), DPP (l)	0 (NEG)	0,05	51	-
13	43	FTT	0 (NEG)	0,11	0,02	0,46	45	D, WL (l)	0 (NEG)	0,06	48	-
14	2	AD	10 (POS)	2,93	0,09	1,86	32	U, A (l)	2 (NEG)	0,07	34	-
15	4,5	severe IC, CD	3 (POS)	0,36	0,44	0,56	19,5	U A (l)	0 (NEG)	0,07	20	-
16	10	AD, FTT, important U A (CMA)	7 (POS)	6,05	0,09	0,03	-	not performed	0 (NEG)	0,09	10	-
17	10	AD	0 (NEG)	0,02	0,14	0,01	11	U (l)	8 (POS)	5,6	12	NS, OS, V, U (l)
18	94	AD, CD, AP, FTT	0 (NEG)	0,06	0,08	0,07	96	AP, D (e)	0 (NEG)	0,04	97	AP, D (e)
19	3	AD, GERD	14 (POS)	3,04	0,08	0,05	-	**not performed - AN (accidental exposure) at 45 m**	0 (NEG)	0,05	47	-
20	15,5	GERD, FTT	2 (NEG)	0,23	0,98	0,76	16	V (l)	0 (NEG)	0,1	16,5	-
21	16	AD, AN (EA)	1 (NEG)	0,60	0,09	2,16	16	AD (l)	0 (NEG)	0,05	17	-
22	9	GERD, generalized important U (CMA)	4 (POS)	2,25	1,23	5,32	-	not performed	0 (NEG)	0,02	10	-
23	4	AD, FTT	15 (POS)	12,20	0,09	0,09	8	AD (l)	0 (NEG)	0,13	10	-
24	58	AD, DPE	0 (NEG)	0,09	0,09	0,09	59	D (e), AD (l)	0 (NEG)	0,01	59,5	-
25	43	GERD, DPE*	5 (POS)	1,57	1,09	0,32	45	GERD (l)	0 (NEG)	0,33	48	GERD (l)
26	7	AD	15 (POS)	17,40	0,08	0,07	-	not performed – generalized U (l) and NS (l) (accidental exposure) at 17 m	4 (POS)	1,51	19	-
27	5	AD	0 (NEG)	0,09	0,09	0,09	10	AD, D (l)	0 (NEG)	0,01	12	-
28	56	**AD, AN (CMA)**	3 (POS)	0,83	0,10	0,03	81	U, V, NS (l)	0 (NEG)	0,01	82	-
29	16	AD, DPE	2 (NEG)	0,13	0,17	0,44	17	V, D, ER (l)	0 (NEG)	0,12	18	-
30	5	AD	8 (POS)	8,71	0,01	0,19	22	U (l)	0 (NEG)	0,02	23	-
31	50	AD, AN (EA)	7 (POS)	0,14	0,02	0,07	51	U (l)	0 (NEG)	0,01	52	-

children suffering from severe CMA-FPIES. Of the 7 children who were intolerant of eHF, only one was also intolerant of DM. Nine children (9.78%) were positive at the DM challenge. Clinical symptoms, none of which were life-threatening, are detailed in Table I. Only 4/9 were sensitised to DM (SPT and/or sIgE positive). A further 14/83 children (16.8%) were sensitised to DM, but none reacted at the FC. 10/11 children with prior CMA anaphylaxis had negative DM IgE tests.

DM consumption

At T_{end}, the period of DM consumption in the 83 children was 1-69 months (mean 15.48 months, median 12). Note that eight patients were only recruited 1-2 months before the end of the study. DM consumption was 200-900 ml/day depending on age (mean and median 300 ml/day). The reasons why children dropped out of the study were: 23/83 acquired a tolerance of CM, after a mean period of DM ingestion of 13 months; in a further 8 cases DM was suspended due to its high cost, after a period of at least 12 months ingestion; a further 4 children (mean age 45 months) no longer wanted to drink DM, after a mean period of ingestion of 15.5 months.

Growth-related data

For the 83 children (49 boys) who regularly and uninterruptedly ingested DM for up to 69 months, the duration of follow-up enabled statistical elaboration to be applied to growth figures for these cases. Table II reports auxological values at T_0 and T_{end}.

55/83 subjects (35 boys) participated in all the scheduled check-ups, at least until T_4. In these patients, negative Z-score values at recruitment, for weight and for length/stature respectively, were found in 44/55 (80.0%) and 36/55 (65.5%) subjects, and a significant increase in Z-score was recorded both for weight ($p<0.001$; $F=17.52$) and for length/stature ($p<0.001$; $F=9.29$) during the entire period of evaluation (Fig. 1).

Nutritional parameters

For 24 children, parental consent was obtained to evaluate nutritional parameters at T_0 and at T_C (median 8 months). Table III shows the parameters for which a statistically-significant difference was found.

Native Electrophoresis and Immunoblotting

Figure 2A shows the separation of DMP in native

Table II. *Growth-related data*

	T_0 Mean (DS)	T_{end} Mean (DS)	Statistical significance
Weight (Kg)	11.00 (3.10)	13.34 (3.09)	
Length/stature (cm)	82.51 (11.64)	91.55 (10.38)	
Z-score for weight	-0.95 (1.15)	-0.57 (1.19)	$p < 0.01$*
Z-score for length/stature	-0.42 (1.15)	0.01 (1.13)	$p < 0.01$*

* *t-test for paired data*

Table III. *Nutritional parameters*

	T_0 Mean (DS) Median (IQR)	T_c Mean (DS) Median (IQR)	Statistical significance
Hemoglobin (g/dl)	11.93 (0.99) 11.80 (1.55)	12.43 (0.95) 12.50 (1.00)	p = 0.008*
Serum iron (µg/dl)	76.78 (43.36) 73.00 (39.50)	89.55 (35.25) 80.00 (42.50)	p = 0.006#
Saturated transferrin (%)	20.78 (9.50) 18.00 (9.50)	23.78 (9.71) 23.00 (13.50)	p = 0.005#
1,25-$(OH)_2$ vitamin D (pg/ml)	73.39 (15.06) 71.20 (14.85)	64.37 (15.69) 65.00 (13.65)	p = 0.019#
Serum prealbumin (mg/dl)	17.62 (4.29) 18.00 (4.50)	19.14 (3.81) 19.00 (6.50)	p = 0.039*

* *t-test for paired data*
\# *Wilcoxon-test for paired data*

conditions. The proteins in the numbered bands were identified by MALDI-TOF MS and are listed in Table IV. Bands 6, 7 and 8, highlighted in bold type in Fig. 2A, are specific to the subjects allergic to DM; (nos. 7, 8, 10, 15 and 17; in bold in Fig. 2B).

Through native electrophoresis, the recognition reactions of the IgE of allergic subjects toward the conformational epitopes of the DMP were pointed up. The five patients allergic to DM (nos. 7, 8, 10, 15 and 17) showed specific reaction bands (bands 6, 7 and 8 in Fig. 2A) that were not found in subjects who were only allergic to CM.

DISCUSSION

The results of this prospective study show the high tolerability (90.2%) of DM in a series of 83 children with proven CMA, larger than previous series. This result confirms those of two recent studies [18,19] on smaller series, in which DM was tolerated respectively by 25/26 (96%) and by 24/25 (96%) recruits. Unlike our series, which included subjects with severe CMA (prior anaphylaxis, acute severe FPIES, children with GI symptoms with malabsorption syndrome and FTT), the children enrolled in the smaller studies only suffered from a mild or moderate form of CMA.

The high percentages of tolerability of DM observed in our subjects, both with IgE-mediated and with non-IgE-mediated CMA, were not dissimilar, suggesting that tolerability of DM is independent of the type of allergic reaction to CMP.

All of the 11 patients with prior anaphylaxis to CM tolerated DM at the FC and during the follow-up. However, in a previous study including five patients with prior anaphylaxis to CM [9] , only one tolerated DM at the challenge. At present no further data are available

Table IV. *Identification of the proteins in bands 1A to 8 in Fig. 2 by MALDI TOF and MALDI TOF/TOF mass spectrometry.*

Band	pI	MW kDa	Protein (Accession number)	Maldi Mass spectrometry Identification matching peptides (coverage %)
1A (fig.2A)	5.78 6.02	25511 25305	Beta-casein precursor (Q9GKK3 Horse) Alpha-s1 casein (Q8SPR1 Horse)	6 (21) 4 (13)
1B (fig.2A)	5.78 6.61	25511 76141	Beta-casein precursor (Q9GKK3 Horse) Serotransferrin precursor (Transferrin) (P27425 Horse)	6 (21) 8 (19)
2 (fig.2A)	6.02 8.32	25305 75420	Serum albumin precursor (Q5XLE4) Lactotransferrin precursor [fragment] (Lactoferrin) (O77811 Horse)	26 (45) 7 (15)
3 (fig.2A)	-	-	Not identified	-
4 (fig.2A)	4.85	18500	Beta-lactoglobulin I precursor (variant B)	18 (81)
4B (fig.2A)	4.79	18528	Beta-lactoglobulin I precursor (P13613)	6 (42)
5 (fig.2A)	4.85 6.61	18500 76141	Beta-lactoglobulin I precursor (variant B) Serotransferrin precursor (Transferrin) (P27425 Horse)	13 (67) 11 (25)
6 (fig.2A)	6.61 4.70	76141 18530	Serotransferrin precursor (Transferrin) (P27425 Horse) Beta-lactoglobulin-2 (Beta-LG-2), (Beta-lactoglobulin II, minor monomeric) (P19647)	11 (29) 7 (52)
7(fig.2A)	4.70	18530	Beta-lactoglobulin-2 (Beta-LG-2), (Beta-lactoglobulin II, minor monomeric) (P19647)	7 (52)
8 (fig.2A)	5.78 6.61	25511 76141	Beta-casein precursor (Q9GKK3 Horse) Serotransferrin precursor (Transferrin) (P27425 Horse)	6 (21) 8 (19)

concerning the tolerability of DM in subjects with prior anaphylaxis to CM; thus, although the results of the present study are promising, the use of DM in these subjects should be contemplated with considerable caution, and the introduction should always be in a hospital setting. Data concerning the tolerability of DM in subjects with severe FPIES due to CMA are also good, but no conclusion may be drawn in this connection, considering the small number of cases.

DM was tolerated in our 83 patients not only at the FC, but for the entire follow-up (mean 15.48 months, median 12), which was sufficiently long to exclude the onset of clinical reactions consequent on primary sensitization to DM.

Of the nine children (9.78%) who were positive at the DM challenge, in no case did DM induce severe systemic reactions of the immediate type at the FC, in line with previous findings [9,18,19] and it caused immediate reactions in only two of our patients out of nine who were positive at FC.

Specific allergometric tests for DM had poor predictivity on both the outcome of the DM oral challenge and the onset of immediate clinical reactions; in particular, the high number of false-positive DM IgE tests might be due to the presence of sIgE primitively directed against epitopes of CMP cross-reacting with epitopes of DMP, without however being responsible for clinical reactions against the latter. DM IgE tests, on the contrary, showed good negative predictivity for the outcome of the DM oral challenge; this was particularly true in the 11 patients with prior anaphylaxis to CM, 10 of whom were not sensitized to DM and none of whom reacted at the FC with DM.

Our patients were highly problematic from the feeding standpoint, since most of them (89%) were affected by multiple FA, leading to the exclusion of staple foods (e.g. egg, wheat, fish) and obliging them to follow a particularly restrictive and monotonous diet: this made it of vital importance to identify a replacement foodstuff for CM, in spite of their age. Unfortunately, current CM substitutes (SF, eHF, RHF and AAF) could not be used. In this connection, the patients recruited for this study were particularly demanding with regard to the palatability of foods, presumably due to their age. DM was found to be a valid alternative including from the standpoint of palatability: its pleasant taste made DM immediately acceptable to all our patients.

Weight and length/stature-gain were evaluated in terms of Z-score. This method evaluates changes in anthropometric parameters associated with the introduction of DM independently of age. The great majority of subjects had negative weight and length/stature Z-scores at enrolment, and showed a significant improvement during the study period (Table II). In particular, the group of patients who completed the follow-up of 12 months presented increases in weight and length/stature Z-score at each checkup after the introduction of DM, as Fig. 1 shows.

The nutritional parameters also showed encouraging results (see Tab. III), although these were available for a smaller number of patients; the diets of all 24 children were particularly restricted, despite their being balanced by the dietician. Diet did not undergo any particular changes between T_0 and T_C, except for the addition of DM. These results, which are in agreement with those of another recent study [19], may in part be attributed to the reported nutritional characteristics of DM. [25,26].

Native electrophoresis of the DMP revealed three peculiar bands present only in the immunoblotting experiments of DM allergic subjects. These immunoreactive bands contained donkey β-casein and β-lactoglobulin II. The lack of pure DM standard proteins made it impossible to perform inhibition experiments with these two cross-rective proteins. This result is interesting in the light of the fact that, for human β-casein, the existence of a conformational epitope cross-reactive with the sIgE for bovine β-lactoglobulin has been reported [27], making it plausible that the only CM protein that can stimulate the production of IgE cross-reactive with the DMP is β-lactoglobulin.

CONCLUSION

It was of great importance to be able to use a palatable and well tolerated substitute foodstuff for our 83 children; despite their restricted diet, DM helped these children to achieve correct growth in terms of height and weight, and normalized the blood-chemistry parameters evaluated, or maintained those parameters within the normal range. A final but no less important aspect is the psychological advantage of being able to use "proper" milk as an alternative to CM, The children either consumed DM as such, or in the form of derivatives (e.g. ice-cream) or of other milk-based foods (e.g. cakes, puddings, home-made biscuits).

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ORIGINAL CONTRIBUTION

Donkey milk consumption exerts anti-inflammatory properties by normalizing antimicrobial peptides levels in Paneth's cells in a model of ileitis in mice

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Abstract
Purpose In this study, we showed the beneficial effects of donkey milk (DM) on inflammatory damages, endogenous antimicrobial peptides levels and fecal microbiota profile in a mice model of Crohn's disease. Nowadays, new strategies of microbiome manipulations are on the light involving specific diets to induce and/or to maintain clinical remission. Interest of DM is explained by its high levels of antimicrobial peptides which confer it anti-inflammatory properties.
Methods C57BL/6 mice were orally administered with or without indomethacin for 5 days and co-treated with vehicle, DM or heated DM during 7 days. Intestinal length and macroscopic damage scores (MDSs) were determined; ileal samples were taken off for microscopic damage (MD), lysozyme immunostaining and mRNA α-defensin assessments. Ileal luminal content and fecal pellets were collected for lysozyme enzymatic activity and lipocalin-2 (LCN-2) evaluations. Fecal microbiota profiles were compared using a real-time quantitative PCR-based analysis.
Results Administration of indomethacin caused an ileitis in mice characterized by (1) a decrease in body weight and intestinal length, (2) a significant increase in MDS, MD and LCN-2, (3) a reduction in both α-defensin mRNA expression and lysozyme levels in Paneth's cells reflected by a decrease in lysozyme activity in feces, and (4) a global change in relative abundance of targeted microbial communities. DM treatment significantly reduced almost of all these ileitis damages, whereas heated DM has no impact on ileitis.
Conclusions DM consumption exerts anti-inflammatory properties in mice by restoring the endogenous levels of antimicrobial peptides which contribute in turn to reduce microbiota imbalance.

Keywords Donkey milk · Antimicrobial peptides · Crohn's disease · Ileitis · Dysbiosis

Abbreviations

CD	Crohn's disease
DAPI	4'6-diamidino-2-phenylindole
DM	Donkey milk
GULDA	GUt low-density array
IBD	Inflammatory bowel disease
MD	Microscopic damage
MDS	Macroscopic damage scores
NSAIDs	Non-steroidal anti-inflammatory drugs
PBS	Phosphate-buffered saline
PCA	Principal component analysis

Introduction

Crohn's disease (CD) is one of the two forms of inflammatory bowel diseases (IBDs). The inflammation in CD patients mainly occurs in the ileum [1]. This pathology is reflected in symptomatic spurts with variable severity and duration, resulting in abdominal cramps, diarrhea, fatigue

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and loss of body weight. The disease flares alternate with phases of clinical remission [2]. Nowadays, there is growing consensus that the ultimate strategy of IBD management is to focus on inducing and maintaining clinical remission [3]. The etiology of CD remains unclear, but data plead for a multifactorial etiology considering genetic, immunological, microbial and environmental factors [4, 5]. Among these factors, a dysbiosis is usually associated with CD and contributes to the initiation and/or perpetuation of chronic mucosal inflammation [6–8]. As compared to healthy subjects, alteration of the gut microbiota profile of CD patients is observed and is notably characterized by an imbalance of the ratios between the two major phyla Firmicutes and Bacteroidetes [9, 10]. Among the Firmicutes phylum, a decrease in *Faecalibacterium prausnitzii* was observed in the ileal mucosa of patients operated for Crohn's ileitis. Interestingly, this ratio was inversely correlated with the risk of postoperative recurrence [11–13]. Moreover, a significant increase in Enterobacteriaceae in IBD patients relative to the healthy population has also been described. In addition to this microbiota imbalance, a dysfunction of Paneth cells has also been reported [14]. These epithelial cells located in the bottom of the ileal crypts are the main source of antimicrobial peptides such as α-defensins or lysozyme [14–16]. These antimicrobial peptides act as innate immunomodulators, leading to the protection against pathogenic invasion. In turn, these peptides allow selective control of inflammation by regulating microbial status in the intestine [17]. A molecular characteristic of patients with ileal CD is a reduction in the expression of α-defensins human defensin 5 and 6 in Paneth cells, resulting in an impaired defense barrier through a reduction in luminal antibacterial host defense capacity [18–21].

Therapeutic manipulation of intestinal microbiota can target selective beneficial bacteria species versus detrimental species. These new strategies of microbiome manipulations are on the light and may involve antibiotics, probiotics, prebiotics and use of specific diets [22–25]. In this way, among natural food matrices, donkey milk presents a high level of antimicrobial peptides and potentially seems a good candidate for reducing ileitis damage. Donkey milk (DM) has important nutritional properties for humans linked to its similar composition to human milk [26, 27]. DM is mainly used for infant nutrition as a natural substitute milk when breast feeding is not possible or to replace bovine milk in diet therapy of patients affected by cows' milk protein allergy [28–30]. In particular, DM presents high levels of antimicrobial peptides such as lysozyme and lactoferrin (20 and 5 % of the total soluble proteins, respectively) that give it antibacterial properties [31], as well as anti-tumoral and anti-proliferative properties on A549 human lung cancer cells [32].

Therefore, the objectives of this study were to characterize using an adapted experimental mouse model of ileitis mimicking CD [33], the effect of a chronic administration of donkey milk on macroscopic and microscopic inflammatory scores as well as on microbiota profile and on endogenous antimicrobial peptides levels contained in Paneth cells.

Materials and methods

Donkey milk

Fresh milks from donkeys located in southwest of France ("Les Ânes d′Autan", Graulhet and "La ferme du Hitton", Biran) were used in this study. Immediately after milking, samples were kept in an ice box (<4 °C) and stored 1 h later in the laboratory at −20 °C until lysozyme activity measurements. These enzymatic activities were measured using an EnzCheck Lysozyme Assay Kit (Molecular Probes E-22013, Thermo Fisher Scientific). The obtained results from these assays allowed application to all the animals orally treated by DM the same total daily activity of lysozyme, i.e., 11800 UI in a total adjusted volume (0.4 ± 0.05 mL). In parallel, to completely eliminate lysozyme activity samples of DM were heated at 140 °C at pH = 9.5 for 40 mn, and checked using the same EnzCheck Lysozyme Assay Kit.

Animals

Eight-week-old male C57BL/6 (weighting 23 ± 0.5 g) (Janvier, Le Genest St Isle, France) was used in this study. Mice were maintained in the pathogen-free animal facility at a constant temperature (21 ± 2 °C) on a 12-/12-hour light/dark cycle. Mice had free access to water and to standard rodent food (Harlan, Global diet 2018 containing crude protein 18.6 %, crude fat 6.2 %, crude ash 5.3 % and crude fiber 3.5 %). Animal care and work protocols were approved by the local ethical committee, according to the EU directive 2010/63/EU (Agreements #Toxcom0143HE).

Experimental protocol

Male C57BL/6 were divided into six groups ($n = 12$ per group), i.e., three control groups (basal conditions) and three indomethacin groups (inflammatory conditions). In both conditions, animals received orally twice per day either vehicle (0.2 mL of PBS) or donkey milk (DM, 11800 UI in 0.2 ± 0.05 mL) or heated DM (0.2 mL). The first treatments were performed 2 h before, during and 2 days after ileitis induction by indomethacin administration. Adapted from Craven et al. [33], ileitis was induced by oral administration of indomethacin diluted in PBS (0.25 mg/mouse) for 5 days. Body weight was evaluated everyday:

the day before and from the beginning of the treatment until 2 days post-indomethacin. Then, animals were sacrificed, and intestinal length and macroscopic damage score were determined. Ileal samples were taken for (1) microscopic damage score (2) lysozyme immunostaining and mRNA α-defensin assessments. Ileal content and fecal pellets were collected for lysozyme activity evaluation. Fecal materials were also taken for microbiota characterization and lipocalin-2 quantification as intestinal inflammatory biomarker.

Mice body weight and intestinal length

The body weight of mice was recorded the day before and followed every day after the ileitis induction by indomethacin administration until the day of sacrifice. The intestine length was measured after the sacrifice.

Macroscopic damage scores (MDSs)

Immediately after sacrifice, the ileum was removed and rinsed with saline. Intestinal damages were scored according to an adapted scale of Wallace et al. [34]. Briefly, the presence of mucosal hyperemia, the intestine wall thickening, the severity and extent of ulceration and necrosis, the tissue adhesion and the occurrence of diarrhea were rated according to a macroscopic damage score ranging from 0 (normal appearance) to 7.5 (severe damages).

Microscopic damage scores (MD)

Pieces of collected ileum samples were fixed in 4 % formaldehyde for 24 h, embedded in paraffin blocks and cut into 5 µm sections for histology analysis. Paraffin sections were stained with hematoxylin and eosin. Microscopic damage scores of inflammation were assessed using a histological grading scale adapted to Fabia et al. [35]. This scale takes into account the leukocyte infiltration, the vascular dilatation, the presence or not of edema, the thickening of the mucosa, the ulceration and the status of the mucus layer. Microscopic damage score is ranging from 0 (normal appearance) to 5 (severe inflammatory damages).

Quantification of fecal lipocalin-2 by ELISA

For quantification of lipocalin-2 (LCN-2) by ELISA, frozen fecal samples were reconstituted in PBS and vortexed for 20 min to get a homogenous fecal suspension as described previously [36]. Then, the samples were grinded using FastPrep and centrifuged for 10 min at 12.000 rpm and 4 °C. Clear supernatants were collected and stored at −20 °C until analysis. LCN-2 levels were measured using the Duoset murine LCN-2 ELISA kit (R&D Systems, Minneapolis, USA).

Immunohistochemistry: lysozyme immunostaining

Sections of ileum segments (5 µm) were hydrated and dipped in a bath of citrate buffer 10 mM, pH 6 at 95 °C, to regenerate antigens. After saturation of the non-specific binding site, sections were incubated successively with antibodies of rabbit anti-lysozyme (1/100) overnight at 4 °C and with antibody of donkey anti-rabbit alexa488 (1/2000). The slides were rinsed with PBS and covered with mounting medium containing DAPI (P36931, Invitrogen). The number of Paneth cells containing lysozyme per crypt was quantified in a blinding manner using a 90i Nikon fluorescence microscope.

mRNA expression by real-time reverse transcription-Polymerase chain reaction (RT-PCR): α-defensins mRNA expression

Total mRNA was extracted from ileum segment with RNeasy mini kit. A total of 500 ng were used to perform reverse transcription during 60 min at 37 °C. The real-time PCR was performed using 25 ng of cDNA in a final volume of 20 µL containing SYBR Green TaqMan Universal PCR Mix. Fluorescence was recorded and analyzed. Analysis of the 18S ribosomal RNA was performed with the TaqMan assay kit control to normalize gene expression overall α-defensins. Specific primers were obtained from Eurogentec (Angers, France): forward, 5′-GGT-GAT-CAG-CAT-ACC-CCA-GCA-TCA-GT-3′; reverse, 5′-AAG-AGA-AAA-CTA-CTG-AGG-AGC–AGC-3′ to amplify the α-defensins. To normalize our values, the reference gene 18S as: forward, 5′-GACCAW-ACA-CGG-GAA-ACC-3′; reverse, 5′-CAA-AGAATC-GCT-CCA-CCA-AC-3′ has been chosen.

GUt low-density array (GULDA) analysis: relative abundance of 20 different bacterial 16S rRNA gene targets in fecal sample from C57BL/6 mice

Total community of DNAs was extracted in a randomized and a blinded manner from fecal samples using mechanical lysis plus column method (ZR Fecal DNA MiniPrep kit Zymo research, Irvine, USA). The DNA concentration was determined using Qubit® ds DNA HS assay kit (life technologies) and adjusted to 1 ng/µL prior to use as a template in qPCR. Change in the abundance of 20 different bacterial 16S rRNA gene targets was obtained using the GULDA approach as previously described [37, 38]. Herein, this method was adapted using only 20 bacterial targets for communities in mice. Indeed, 31 bacterial targets described in the assay were not detected in mice. According to this method, the universal bacterial primer set U1 was included as the reference gene (four technical

replicas of each amplification). Each 384-well PCR plate (MicroAmp optical reaction plates, Applied Biosystems, Naerum, Denmark) accommodated simultaneous analysis of four DNA samples per group (control vs indomethacin vs control + donkey milk vs indomethacin + donkey milk) in duplicate. Quantitative real-time PCR was performed on a ViiA7 from Applied Biosystems in a total volume of 5 µL containing 2.5 µL 2 × Power SYBR Green PCR Master Mix (Applied Biosystems), 0.18 µL of each primer (10 µM), 1 µL template DNA and 1.14 µL nuclease-free water. Liquid handling was performed with a Bravo platform (Agilent Technologies, Santa Clara, USA). Following the previously described thermocycling program, the raw fluorescence data recorded by the ViiA7 RUO Software were exported to the LinRegPCR program to perform baseline correction, to calculate the mean PCR efficiency per amplicon group and to calculate the initial quantities. No (arbitrary fluorescence units) for each amplicon. The relative abundance of the 20 specific amplicon groups was obtained by normalization to the No value obtained for the universal bacterial amplicon group determined in the same array (No, specific/No, universal). A limit of detection of 10^{-6} (*No*, specific/*No*, universal) was set, and samples below this limit were set to 5.10^{-6}. The normalized No-values (log10 transformed) obtained from each amplicon group were used as input for multivariate principal component analysis (PCA) using FactoMineR (version 1.31.3).

Lysozyme activity in both ileal and fecal materials

After sacrifice, ileal contents and feces were collected. The samples were stored at 4 °C. Each sample was mixed and homogenized in 500 µL of kit buffer. After centrifugation (10 min, 8000 rpm, 4 °C), pellets were discarded and supernatants were filtered by 0.8 µm-sized syringe filters.

The activities of lysozyme contained in these supernatants were measured as previously described using the EnzCheck Lysozyme Assay Kit (E-22013). To normalize the values obtained, a protein assay was performed using the protein assay kit (Interchim). Results were expressed in lysozyme units of activity/mg protein.

Data analysis

Data are expressed as mean ± SEM. For statistical analysis, GraphPad prism (GraphPad, San Diego, CA) was used. Multiple comparisons within groups were performed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. Statistical significance was accepted at $p < 0.05$. For microbiota data, statistical analysis for comparison between the relative abundance for specific amplicon groups was performed using the GraphPad prism software. Indicated p values <0.05 refer to significance using one-way analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparison Test.

Results

Effect of donkey milk on body weight and intestinal length

In basal conditions, after 7 days of oral administration of DM, animals did not show any difference in body weight nor in intestinal length compared with control mice (Fig. 1a, b). Body weight loss started 24 h after ileitis induction by indomethacin administration, to reach ≈5 %

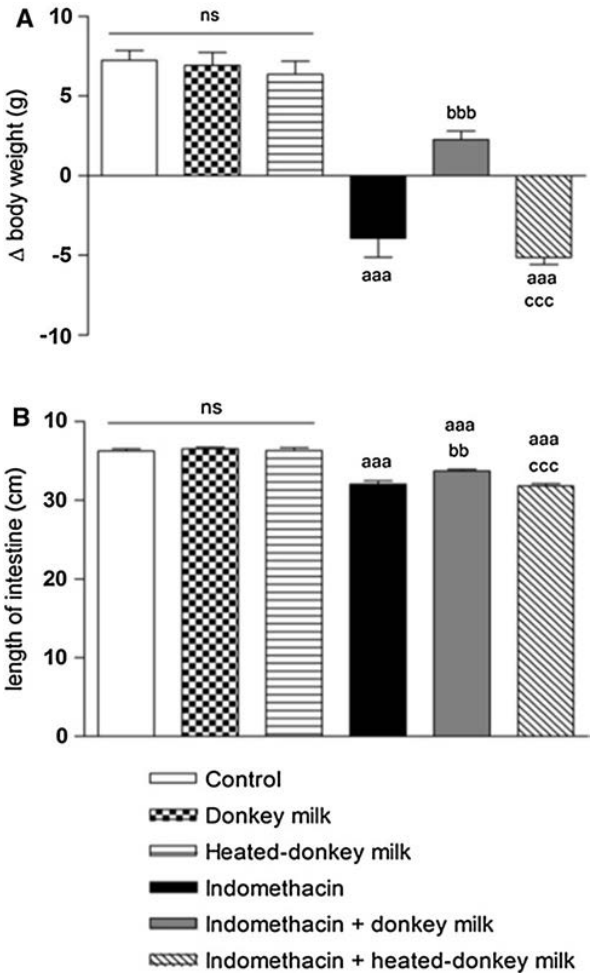

Fig. 1 Effect of donkey milk treatment on **a** body weight and **b** length of intestine. Values are expressed by mean ± SEMs, $n = 12$. *ns* nonsignificantly different: $p > 0.05$. *a*, *aa*, *aaa* $p < 0.05$, 0.01, 0.001, respectively, vs Control. *b*, *bb*, *bbb* $p < 0.05$, 0.01, 0.001, respectively, vs Indomethacin. *c*, *cc*, *ccc* $p < 0.05$, 0.01, 0.001, respectively, vs Indomethacin + Donkey milk

on day 7 post-indomethacin compared to non-inflamed mice (Fig. 1a). A significant reduction ($p < 0.05$) in intestinal length was measured on day 7 post-indomethacin (Fig. 1b). Under inflammatory conditions, mice treated orally with DM showed a significant reduction in both body weight loss as well as a significant less reduction in intestinal length compared to indomethacin ileitis mice (Fig. 1a, b). Interestingly, heated DM treatment failed to reverse the deleterious effects of indomethacin on these two parameters, i.e., body weight and intestinal length (Fig. 1a, b).

Donkey milk treatment attenuates the severity of indomethacin-induced ileitis

In basal conditions, PBS, DM and heated DM did not induce inflammation. Indomethacin administration resulted in ileum inflammation reflected by ileal ulcerative areas, hyperemia, tissue adhesion and diarrhea, leading to a significant increase $p < 0.001$ in MDS 7 days after ileitis induction compared with non-ileitis mice (Fig. 2a). In the same way, compared with control mice, indomethacin administration promoted a drastic increase ($p < 0.001$) of MD scores (Fig. 2b) illustrated by leukocytes infiltration into the mucosa, thickening of the mucosa and ulceration (Fig. 2d). Moreover, fecal LCN-2 levels were significantly increased in inflammatory conditions (Fig. 2c). Oral DM treatment significantly decreased fecal LCN-2 and both ($p < 0.001$) MDS and MD scores. Again, oral heated DM treatment had no effect on the inflammatory damages induced by indomethacin administration (Fig. 2).

Donkey milk treatment restored the antimicrobial peptides contained in Paneth's cells in ileitis mice

No modification of the number of Paneth cells was observed in ileal tissue sections (Table 1). In basal

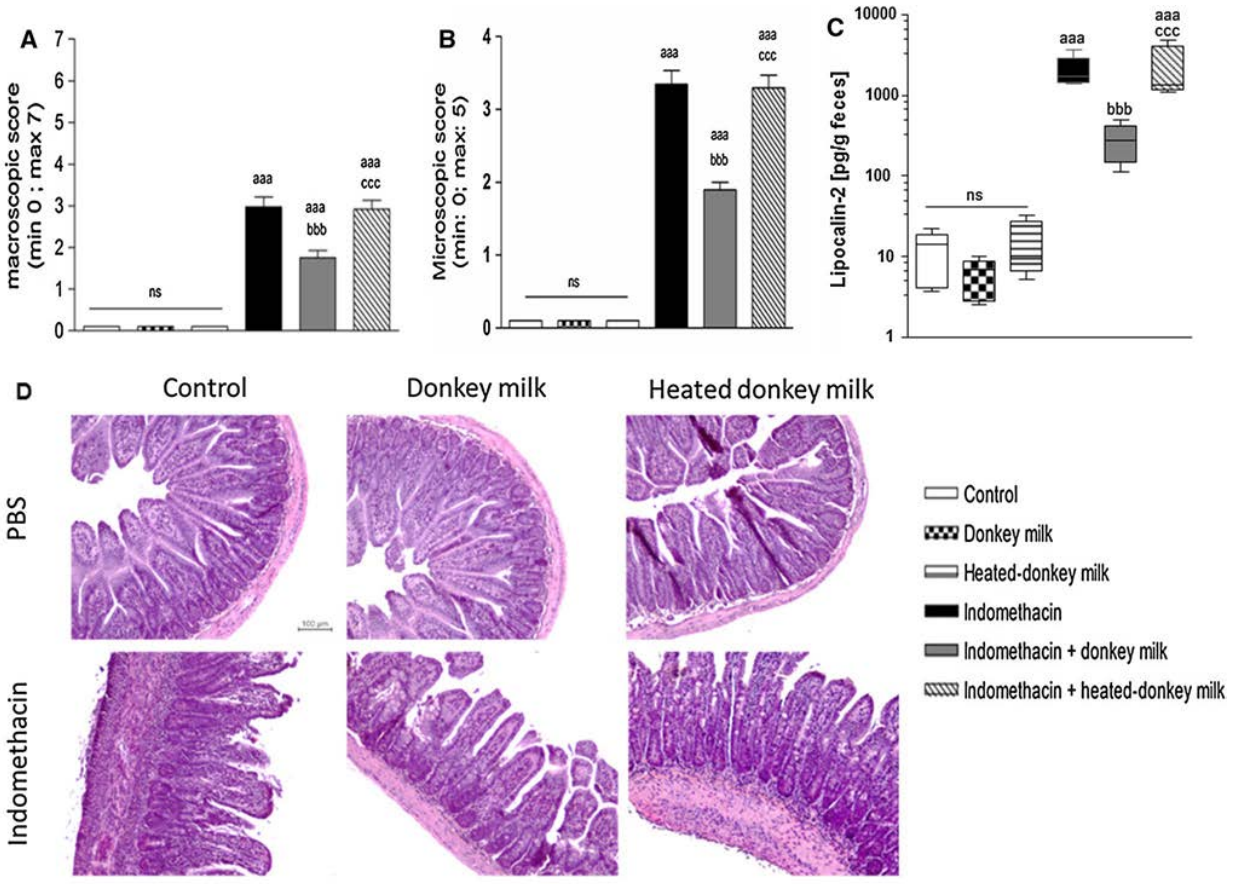

Fig. 2 Effect of donkey milk treatment on **a** macroscopic damage scores, **b** microscopic damage scores, **c** fecal lipocalin-2 and **d** histological illustrations. Values are expressed by mean ± SEMs, $n = 12$. *ns* nonsignificantly different: $p > 0.05$. *a*, *aa*, *aaa* $p < 0.05$, 0.01, 0.001, respectively, vs Control. *b*, *bb*, *bbb* $p < 0.05$, 0.01, 0.001, respectively, vs Indomethacin. *c*, *cc*, *ccc* $p < 0.05$, 0.01, 0.001, respectively, vs Indomethacin + Donkey milk. **d** shows histological profiles with all the treatments

conditions, treatment with DM did not change the mRNA levels of α-defensins compared with control (PBS). In contrast, DM intervention significantly increases lysozyme levels contained in Paneth's cells. In ileitis mice, indomethacin induced a decrease of 55 % of α-defensins mRNA expression and a significant decrease ($p < 0.001$) in lysozyme levels contained in Paneth cells compared with control animals (Fig. 3a–c). In ileitis animals, donkey milk treatment significantly restored ($p < 0.001$) the expression and the levels of these two antimicrobial peptides (α-defensins and lysozyme) versus indomethacin animals ($p < 0.001$). No significant difference on these two antimicrobial peptides expression or levels in Paneth's cells between animals treated with indomethacin plus heated DM versus indomethacin was observed.

Table 1 Effect of each treatment on the number of Paneth cells

	Control	Donkey milk	Heated donkey milk	Indomethacin	Indomethacin + donkey milk	Indomethacin + heated donkey milk
Mean	6.50 ± 0.31	6.33 ± 0.47	6.58 ± 0.38	6.33 ± 0.35	6.67 ± 0.28	6.41 ± 0.39

No significant difference between each treatment. $n = 12$ per group

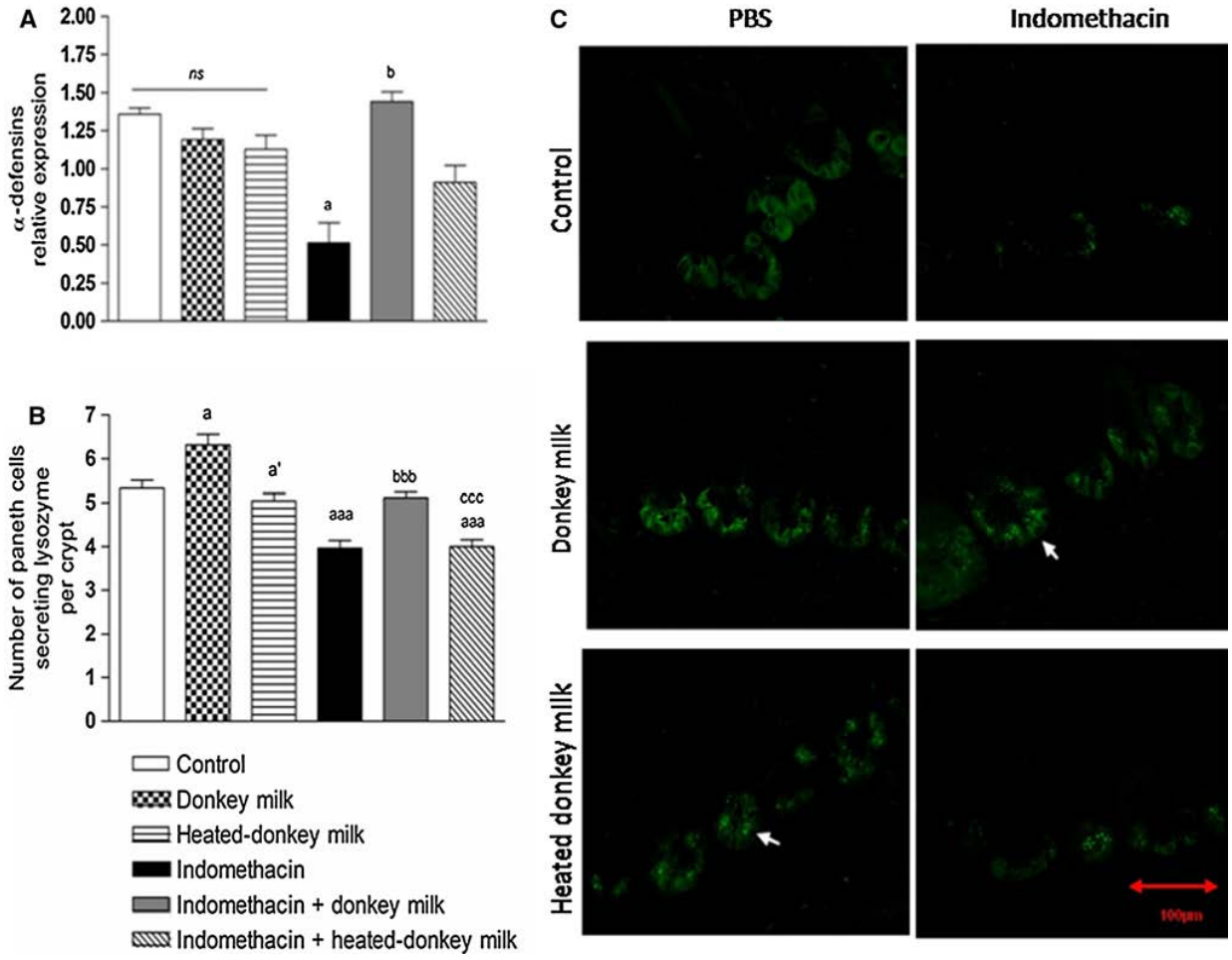

Fig. 3 Effect of donkey milk treatment on antimicrobial peptides. **a** Relative expression of α-defensins by qPCR. **b** Number of Paneth cells secreting lysozyme per crypt. **c** Lysozyme immunostaining (*green*) in Paneth cells of mice according to treatments. The *arrows* are pointing lysozyme in Paneth cells. Values are expressed by mean ± SEMs, $n = 12$. *ns* nonsignificantly different: $p > 0.05$. *a*, *aa*, *aaa* = $p < 0.05$, 0.01, 0.001, respectively, vs Control. *a'* $p < 0.05$ vs Donkey milk. *b*, *bb*, *bbb* $p < 0.05$, 0.01, 0.001, respectively, vs Indomethacin. *c*, *cc*, *ccc* $p < 0.05$, 0.01, 0.001, respectively, vs Indomethacin + Donkey milk

Effect of donkey milk treatment on lysozyme activity contained both in ileal luminal content and in fecal pellets

In basal conditions, no impact of the different treatments applied (PBS, DM or heated DM) was observed on lysozyme activity recovered both in ileal luminal contents and in fecal materials (Fig. 4a, b). Indomethacin administration significantly decreased ($p < 0.01$) lysozyme activity in the feces compared with control mice (PBS) (Fig. 4c). In mice submitted to ileitis and treated with DM, a higher significant lysozyme activity was assessed both in luminal ileal content as well as in fecal material compared with animals treated with indomethacin. The exogenous oral supply by heated DM had no impact on ileal and fecal lysozyme activity decrease induced by indomethacin, i.e., similar values with indomethacin group were observed.

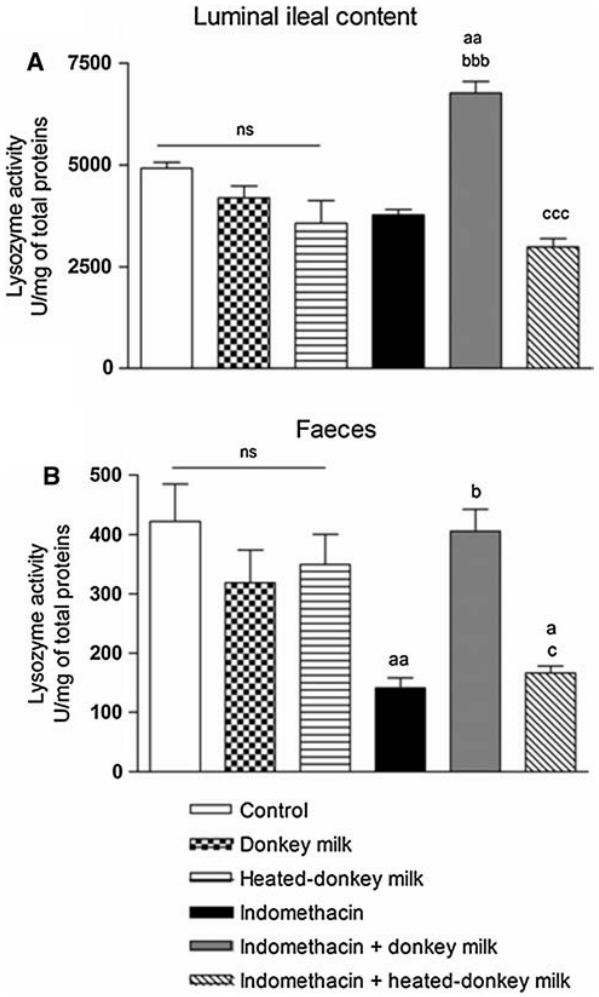

Fig. 4 Effect of donkey milk treatment on lysozyme activity in **a** ileal luminal content and **b** feces, expressed per mg of total proteins. Values are expressed by mean ± SEMs, $n = 12$. *ns* nonsignificantly different: $p > 0.05$. *a*, *aa*, *aaa* $p < 0.05$, 0.01, 0.001, respectively, vs Control. *b*, *bb*, *bbb* $p < 0.05$, 0.01, 0.001, respectively, vs Indomethacin. *c*, *cc*, *ccc* $p < 0.05$, 0.01, 0.001, respectively, vs Indomethacin + Donkey milk

Effect of Donkey milk on dysbiosis observed in ileitis mice

In basal condition, DM treatment had no significant impact on the fecal microbiota profile compared to control group (Fig. 5a and Supplemental Fig. 1). In contrast, Indomethacin induced a significant variation of fecal targeted microbial communities compared to control animals (Fig. 5a and Supplemental Fig. 2). On the dimension 2 of the score plot that represents 17.76 % of the total variance, treatment conditions of mice explained 26 % of variability of the individual coordinates on this dimension ($\eta^2 = 0.22$; $p = 0.02$), and only the coordinate of indomethacin group was statistically different from the others ($p = 0.006$). Interestingly, DM administration to indomethacin-treated mice led to a partial migration of individuals from right to left along dimension 2 compared to indomethacin-treated mice (Fig. 5a). The loading plot indicated that Clostridium cluster XIVa was the bacterial community the most negatively correlated with dimension 2 (correlation = –0.76, $p = 4.7e^{-8}$), whereas *Akkermansia muciniphila* and *Enterococcus spp.* were the two species the most positively correlated with this dimension (correlation = 0.55, $p = 4.88e^{-4}$) (Fig. 5b–d). Regarding their respective relative abundance, increase in *A. muciniphila* abundance that resulted from the indomethacin treatment ($p < 0.001$) was completely reversed by administration of DM ($p < 0.05$) (Fig. 5c and Supplemental Fig. 3). Similarly, the decrease in abundance of Clostridium cluster XIVa resulting from the indomethacin treatment ($p < 0.01$) was limited by DM administration, whereas bacteria belonging to this cluster such as *C. coccoides* and *Eubacterium rectale* were not affected by DM in basal conditions (Fig. 5e and Supplemental Fig. 3).

Discussion

Crohn's disease is a chronic inflammatory bowel disease occurring most commonly in the ileum [39]. The pathogenesis of CD is partially attributed to an intestinal microbiota imbalance that mediates and/or participates in immune-mediated intestinal inflammation in genetically predisposed subjects [40]. Nowadays, therapeutic strategies are focused on maintaining or inducing clinical remission via modification of the intestinal bacteria using antibiotics, probiotics, prebiotics, a diet or a combination of all these approaches [22]. In this study, we show that a chronic oral intervention with a natural food matrix containing high levels of

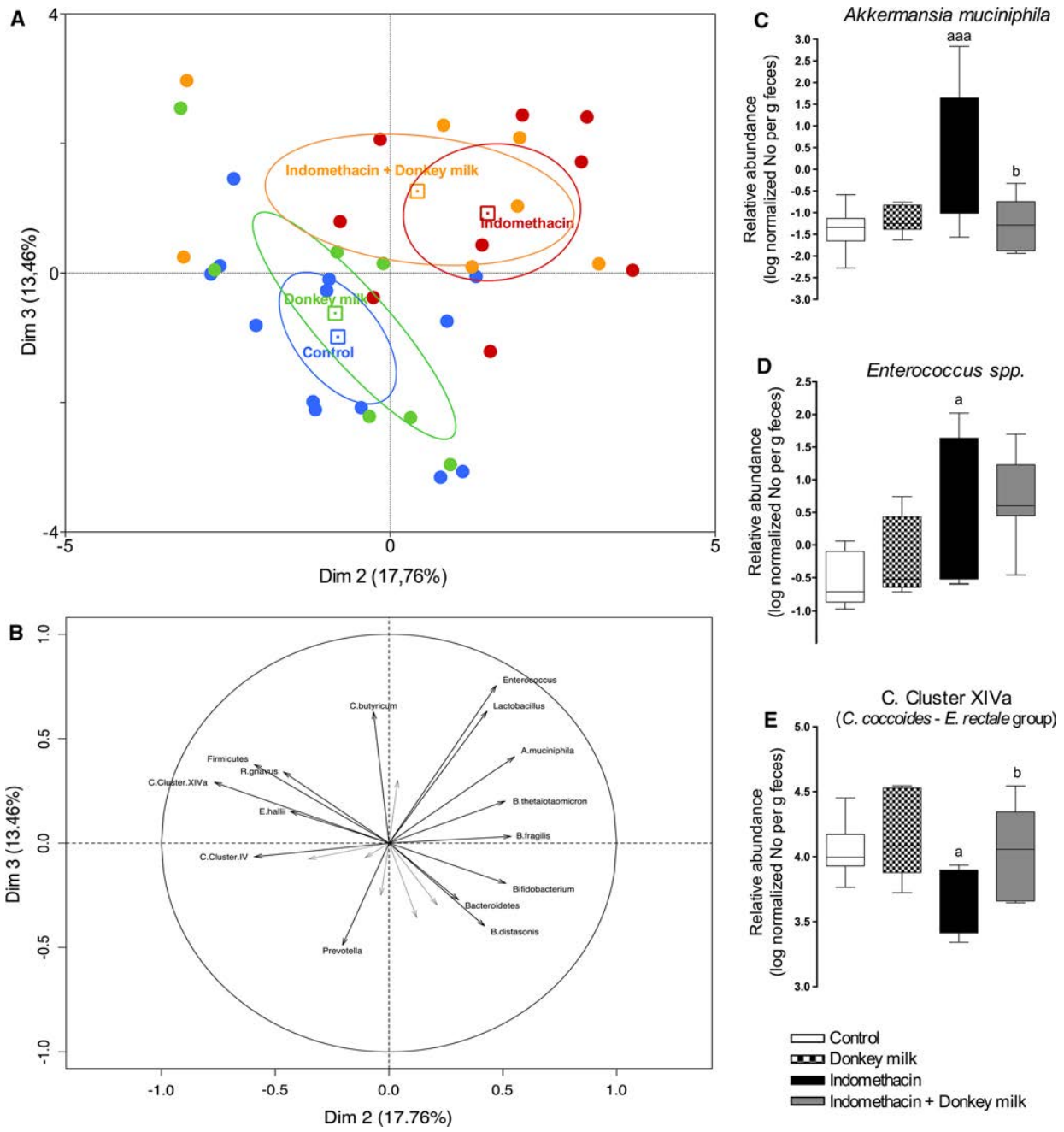

Fig. 5 Effect of donkey milk treatment on fecal microbiota. **a** Principal component analysis (PCA) of microbial communities, score plot. **b** Loading plot. Relative abundance of **c** *Akkermansia muciniphila*, **d** Enterococcus spp., **e** Clostridium *cluster XIVa*. Values are expressed by box plots, control $n = 13$, donkey milk $n = 7$, indomethacin $n = 9$ and indomethacin + donkey milk $n = 7$. *ns* nonsignificantly different: $p > 0.05$. *a*, *aa*, *aaa* $p < 0.05$, 0.01, 0.001, respectively, vs Control. *b*, *bb*, *bbb* $p < 0.05$, 0.01, 0.001, respectively, vs Indomethacin

lysozyme, i.e., donkey milk exerts anti-inflammatory properties in a mouse model of ileitis. This effect is associated with a normalization of antimicrobial peptides (lysozyme and α-defensin) levels contained in Paneth's cells as well as a reduction in the dysbiosis associated with ileitis.

Several nutritional components are able to modify metabolic homeostasis and inflammatory state [41, 42]. Linked to its physicochemical properties and its nutritional value, donkey milk (DM) has recently been suggested as a healthy dietary factor for infants, adults and elderly [26, 43–45].

This food matrix is low in fat but high in polyunsaturated fatty acids (PUFAs) [46], contains high levels of growth factors [47]. It also hosts *Lactobacillus plantarum* that can be considered a probiotic strain [48]. Interestingly, DM also presents high levels in antimicrobial peptides similar to those contained in human milk e.g., lactoferrin (2 g/L) and lysozyme (1–4 g/L) [26, 45]. Therefore, in the present study, anti-inflammatory properties of DM were evaluated after a chronic daily oral administration at a constant enzymatic activity of lysozyme (11800 UI/mice). This activity of lysozyme was contained in a total volume of 0.4 mL (0.2 mL twice daily) and considered relevant, since it corresponds to the equivalent of a daily drinking glass of milk for human (100 mL) [49].

Short-term medication with non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin medication causes a variety of damage to the small intestine, including bleeding, ulcerations and perforations [50, 51]. Further, NSAIDs induce no damage in the small intestine in germ-free rats [52] as well as in animals treated with antibiotics for Enterobacteria suppression [53]. In humans, these observations are largely corroborated, since luminal factors such as bacteria are well described to interact with NSAID to promote an increase in intestinal permeability, a key indicator in NSAID-induced intestinal injury of the distal part of the intestine [54, 55]. Moreover, ingestion of NSAIDs is known to be involved in clinical IBD risk relapse [56]. In mice, oral administration of high dose of indomethacin (1 mg/mouse/day for 3 days) induces ileal inflammation via alteration of the intestinal microbiome mimicking conditions seen in chronic and relapsing CD [33]. In our study, we demonstrate that oral treatment with a lower dose of indomethacin (0.25 mg/mouse for 5 days) also promotes ileitis in mice. A drop of body weight, reduction in the length of the small intestine, as well as a drastic increase in fecal lipocalin-2 and both macroscopic and microscopic damage scores characterizes this inflammatory state. Our study shows that oral DM treatment prevented all these ileitis parameters. As described before, DM is a complex matrix containing several mediators such as interferon γ (IFNγ), lysozyme, lactoferrin, TGF-β and even lactic flora which can participate synergistically in the anti-inflammatory properties of DM. For instance, IFNγ is known to stimulate the antimicrobial activity of macrophages, cytotoxic T cells and natural killer cells (NK) cells [57]. Lactoferrin also contributes to the antibacterial and anti-tumoral properties of DM [44]. *Lactobacillus plantarum* found in DM was recently described to produce bacteriocins promoting bactericidal effects on different strains e.g., *Listeria monocytogenes*, *Lactobacillus curvatus, Enterococcus faecium,* in vitro [48]. Moreover, the lactic flora present in DM plays an anti-inflammatory role through release of nitric oxide [43]. In a previous study, we demonstrated the anti-inflammatory effect of *Lactobacillus farciminis* on a model of colitis induced by TNBS in rats, via the endogenous production of NO by this probiotic strain [58]. Herein, to counteract the enzymatic activity of lysozyme, heated DM (140 °C at pH = 9.5 during 40 mn) was used. Interestingly, heated DM had no impact on the ileitis induced by indomethacin. However, we cannot rule out that the heat treatment applied for degradation of lysozyme has as effect on the other biocompounds contained in DM, and we speculate that the anti-inflammatory effect of DM is linked to a reduction in the antimicrobial properties of DM mediated by lysozyme (1–4 g/L) which reduces dysbiosis. For a long time, lysozyme, a small enzyme, was only known for its antimicrobial activity against Gram-positive bacteria due to its muramidase activity. However, several studies suggest other mechanisms of action against both Gram-positive and Gram-negative bacteria, such as perturbation of DNA or RNA synthesis and membrane permeabilization [59–61].

Therefore, we investigated the effect of DM on the fecal microbiota profile in our model. In basal conditions, compared to control animals, treatment with DM has no effect on the targeted microbial populations which we analyzed. We did observe a clear dysbiosis in indomethacin-induced ileitis. This dysbiosis is illustrated by a reduction in the Firmicutes *Clostridium coccoides* and both a significant increase in Enterococcus spp and *Akkermansia muciniphila* in the feces of ileitis mice. Both a decrease in Firmicutes as well as a significant increase in Enterococci are reported in fecal material coming from CD patients compared to healthy subjects [62–64]. Concerning *Akkermansia muciniphila* only few data make a link between this microorganism and intestinal inflammation. Ganesh and collaborators show that commensal *A. muciniphila* exacerbates *S. typhimurium*-induced intestinal inflammation [65]. IBD often causes increased mucus content in feces. *A. muciniphila* is known to exert mucolytic properties in vitro [66] and to be involved in "a positive feedback loop" by stimulating mucus renewal [67]. Healthy colonocytes produce a mucus layer rich in O-acetylated sialic acids [68]. In contrast, colonic mucin from UC patients is characterized by reduced sulfatation and changes in the degree of mucin glycosylation [69]. Recently, an increased binding capacity of *A. muciniphila* to mucin from UC patients has been shown compared to healthy mucin [70]. Although the chemical changes to mucin were not evaluated in this study, the data reported below allow us to suggest a modulation of the composition of mucus in our ileitis model leading to an increased ability of *A. muciniphila* to bind to this mucus. Interestingly, we show that a chronic treatment with DM in part normalizes both Firmicutes *Clostridium coccoides* and *A. muciniphila* populations in fecal material of ileitis mice, partly counteracting the dysbiosis caused by ileitis. These data suggest that the improvement in symptoms of ileitis

observed after oral DM intervention may be related to the restoration of a healthy microbiome.

Concomitantly, in our model of ileitis, we found a significant reduction in endogenous antimicrobial peptides contained in Paneth cells illustrated by a 55 % reduction on mRNA expression of α-defensins and lysozyme levels without affecting Paneth cell numbers present in the ileal crypt versus control. Paneth cells play a crucial role in mucosal defense and were shown to be functionally impaired in IBD, particularly in patients with CD [21, 71]. For example, compared to healthy subjects, a decreased expression of α-defensin 5 and 6 in ileal Paneth cells from CD patients has been described [19, 20, 72]. Moreover, in a model of ileitis, a significant loss of lysozyme in the base of ileal crypts of SPF-TNF^{deltaARE} mice was recently described, while number of UEA-1 positive cells remained the same [6]. Taken together, these data illustrate that in our model, indomethacin-driven ileitis is mediated by a reduction in Paneth cell function rather than number. In our model, DM treatment restored the expression of α-defensins and lysozyme levels in Paneth cells in ileitis mice to physiological levels. These results suggest that DM consumption leads to a restoration of the functionality of Paneth cells. In physiological conditions, antimicrobial peptides produced by Paneth cells are released into the intestinal lumen where they play a central role in the regulation of the intestinal microbiome [73]. Interestingly, in our study we demonstrate that DM treatments in basal condition significantly increase lysozyme levels contained in Paneth cells. However, compared with control no difference in basal condition was observed in lysozyme activity in both ileal luminal contents and in fecal materials (Fig. 4). Taken together, all these data suggest that in physiological conditions, oral DM treatment enhances the intestinal innate immunity representing by increased storage of lysozyme in Paneth cells. By this way, DM may reinforce host defense against intestinal inflammation such as ileitis. Indeed, we show that DM treatment increases the activity of lysozyme both in ileal luminal content and in feces of ileitis mice. In contrast, heated DM fails to reverse the defect of Paneth cell functionality observed in ileitis animals and promotes a decrease in lysozyme activity in ileal luminal content and in fecal material. Even if we cannot exclude a direct effect of the lysozyme activity coming from DM, all these results indicate a better functionality of Paneth cells, suggesting that DM can reinforce Paneth cell-mediated innate immunity, limiting development of ileitis by counteracting the imbalance of intestinal microbiota.

In summary, consistent with the therapeutic modification of the intestinal microbiota as a new strategy for maintaining or inducing clinical remission in CD patients [22], this study allows to propose donkey milk as a helpful dietary intervention used to maintain/extend the remission periods in CD patients by restoring the endogenous levels of lysozyme and α-defensins in Paneth's cells which in turn contributes to reducing dysbiosis related to ileitis.

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Compliance with ethical standards

Conflict of interest Sophie Yvon, Maïwenn Olier, Mathilde Leveque, Gwenaëlle Jard, Helene Tormo, Djamila Ali Haimoud-Lekhal, Magali Peter, Hélène Eutamène have no conflict of interest.

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Onalat

EUROLACTIS

Why Donkey's milk?

Donkey's milk is an age-old natural product used in cosmetic and healthy beverage since ancient times. Today's scientific community has inherited this tradition and considers Donkey's milk to have the closest composition to that of human mother's milk.

"Onalat© Powder" is obtained by process known as "freeze-drying" or cold drying. This process makes it possible to withdraw the water contained in milk by "sublimation" in order to make it more stable and to facilitate its conservation. Sublimation is the passage of an element of the solid state directly into a gas state without passing by the liquid one: which has many advantages compared to the other drying or conservation processes.

Compared to other types of milk…

Donkey's milk has some unique characteristics, for example:

- high in vitamin C (2.1 mg/100 ml)
- up to 3 times more Omega 3
- ideal ratio of Omega 3/Omega 6
- significantly more immunoglobulin
- up to 4 times less fat
- up to 3 times less sodium (similar to human milk)
- a higher calcium/phosphorus ratio (similar to human milk)
- contains lysozyme (considered for good antibacterial action)
- significantly less casein (very high-allergen proteins): inoffensive trace of Alpha-S2 casein
- naturally hypoallergenic

100 ml of reconstituted milk contain:

Proteine Protein Protéines Eiweiss	Lipidi Lipids Lipides Lipide	Glucidi Carbohdrate Glucides Saccharide	Calcio Calcium Calcium Kalzium	Fosforo Phosphorus Phosphore Phosphor
1.7 g	0.94 g	7.2 g	86.8 mg	52.8 mg

Lattosio Lactose Lactose Laktose	Omega 3+6 * Omega 3+6 * Omega 3+6 * Omega 3+6 *	Energia Energy Energie Energie KJ/100ml	Energia Energy Energie Energie KCal/100ml
7.0 g	169 mg	165.8 KJ	39.1 Kcal

*(poly unsaturated fatty acids: Omegas 3 and 6)

Child growth

Donkey's milk is a naturalfood supplement that can stimulate relevant biological functions during bottle-feeding with formulated or vegetable milk (cow's milk derivatives, soya, rice, etc.). It can also be used during the development period, when children need calories but also a whole series of substances that help building healthy bones and organs. Donkey's milk contains:

- Whey milk proteins with biological functions in the native status (antimicrobial and antiviral general defence system);
- Proteins associated with fat globule membranes with specific biological functions: anti-rotavirus defence properties;
- Peptides originating from digestion of casein with specific biological functions:
  > Opioid function (promotes post-meal sleep),
  > Antihypertensive function,
  > Calcium and phosphorus capture and assimilation.
- Lactose: high content (as much as in human milk), with the following functions:
  > energetic;
  > helps calcium assimilation;
  > pre-biotic effect;
  > source of galactose.
- Naturally hypoallergenic proteins for mother and child.

From 6 months

Recommended dosage as supplement

From 6 to 12 months: supplement a meal (preferably dinner) with Onalat© Powder (3 measuring cups = 10-12 gr, in 100 ml of water).
Over 12 months: every milk feed can be substituted with Onalat© (3 measuring cups = 10-12 gr, in 100 ml of water).

Warning:
not appropriate for lactose intolerants

About Eurolactis & Partners

Eurolactis was created in Switzerland and is the first international vertically integrated supply chain entirely dedicated to Donkey's milk production. With more than 880 Donkeys in 2011, our livestock benefits from optimal living conditions in free half stabling.

Donkey's milk production begins the day after the birth of the foal, but it is necessary to wait a period of one month before milking the donkey mare. Milking shedonkeys requires specific and delicate techniques and is done mechanically to respect strict EU regulations.

Donkey's milk is fragile, and thus, Eurolactis has studied and selected the best way to preserve the precious milk nutriments in freeze-dried powder. Eurolactis is also the supplier of large European food and cosmetics companies in France, Italy, Switzerland, etc. We benefit from strong partnerships with institutions that have more than 15 years experience in breeding and precise Zootechnical skills, as well as the relevant scientific knowledge.

The vertically integrated process directs our value chain, and makes us the leader in this sector. Each partner involved in our supply chain gains a significant financial benefit, such as ensuring better and more competitive access to our products to the final consumer.

AGRICULTURAL. The project is part of an ambitious project which involves economically sustainable collaboration.

MEDICAL & PEDIATRIC. Donkey's milk (DM) is naturally hypoallergenic and is tolerated by people who are sensitive and allergic to cows' milk protein. Likewise, the use of DM as a supplement during breastfeeding has increased considerably.

FOOD. Very similar to human breast milk, DM has all the necessary nutritive substances to confer healthy and desirable values, with a low casein and fat content.

COSMETIC. Donkey's milk is rich in virtues, and stories about its power to make women beautiful have existed since Ancient Times (visit **www.calinesse.com**).

High-performances

Nutrition is essential for everyone, but it becomes vital for those who practice sports or who need to have a high performance level all day long.

- Men and women who practice sports that require strength with short bursts of speed and action have high carbohydrate needs. They require sufficient glycogen to provide them with energy during sustained efforts, combined with a reduced intake of high quality fats;
- In this perspective, breakfast including **approximately 200 ml of Donkey's milk** instead of traditional cows' milk offers significant benefits in terms of increased lactose intake (7% in Donkey's milk);
- Conversely, Donkey's milk contains only about 1% fat (3.5% in whole cows' milk and 1.5% in semi-skimmed milk) which makes Donkey's milk of higher quality in terms of digestibility;
- Out of the total fatty acids, compared for example with cows' or goats' milk, Donkey's milk contains only 58% of saturated fatty acids, of which approximately half are short to medium chain (MCT);
- High proportion of MCT in Donkey's milk with smaller fat globules makes it more bio-available and digestible;
- The particular composition of the protein fraction of Donkey's milk also contributes to improved digestibility and assimilation compared with cows' or goats' milk;
- The casein content is low enhancing the presence of serum protein; this results in a more friable milk coagulation that can be processed more rapidly by the gastric juices.

Assimilated in the food and dietetic program of GEOX-TMC Cycling Team.

Equilibrium food

Some nutritional elements that are particularly important during the adult age are those favouring the maintenance of personal performance, while reducing the risk of cardiovascular disease, bone fractures and strength loss. Proteins, polyunsaturated fatty acids and lactose can enhance calcium assimilation and are known to be among the most important nutritional components.

- Freeze-dried Donkey's milk contains 17% protein and provides a well-balanced contribution of caseins and whey milk proteins, with a high content of essential amino acids;
- The high proportion of polyunsaturated fatty acids in the total amount of fatty acids present in Donkey's milk, and in particular, the high content of Omega 3, reduces the risk of potential damage to the cardiovascular system;
- The high content in lactose of Donkey's milk, about 7%, contributes to the calcium assimilation process, therefore reducing the risk of bone fragility;
- One aspect which makes Donkey's milk particularly useful for the elderly (especially compared with cows' milk) is the low saturated fatty acid content: 1/4 less than for cows' milk and in addition, Donkey's milk contains mainly "Medium Chain Fatty Acids (MCFA)", whereas ruminant milk contains "Long Chain Fatty Acids (LCFA)". MCFAs are easier to assimilate than LCFAs, hence better from a nutritional standpoint;
- Less fat, easier to digestive.

Recommended dosage

It is possible to substitute the entire daily consumption of other types of milks with Onalat® Donkey's milk (3 measuring cups = 10-12 gr, in 100 ml of water).

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