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The neglected nutrigenomics of milk: What is the role of inter-species transfer of small non-coding RNA?

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Abstract:	The characterization of small non-coding RNA (sncRNA) in food has become part of the field as a promising field of nutrigenomics. Milk contains sncRNA that are protected by extracellular vesicles which makes them resistant to digestive processes and possibly absorbable by the human gut. Due to the high conservation of sncRNA, these molecules might mediate inter-species gene expression regulations, opening numerous applications in the field of human nutrition. These include the modulation of sncRNA milk profile through diet, both in humans and dairy animals, livestock rearing methods, food technology, but also the production of infant formulas or the usage of sncRNA as biomarkers. SncRNA contained in milk might contribute to the elucidation of the long-term effects of milk consumption in the human diet, confirming the application of nutrigenomics in both health promotion and food production areas. The main aim of this mini-review is to introduce this aspect of nutrigenomics illustrating both promising aspects and pitfalls.
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To Prof. Editors-in-Chief
Jian Chen
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Food Bioscience

Camerino, October 1st, 2020

Dear Editors,

we are pleased to resubmit the review article entitled ***"The neglected nutrigenomics of milk: what is the role of inter-species transfer of small non-coding RNAs?"*** by prof. Rosita Gabbianelli and me. We revised the manuscript according to the Editor's requests.
Thank you for your efforts in improving the quality of this manuscript.

Yours Sincerely,

Laura Bordoni, PhD

The authors reviewed the manuscript according to the Editor’s suggestions.
All the issues have been accomplished.

HIGHLIGHTS

- SncRNA are present in both plant- and animal-based food, especially in milk;
- Exogenous sncRNA might regulate immune- and development-related processes;
- Local effects and microbiome modulations are the major focus of sncRNA research;
- Environment (i.e., diet) and health status modulate the milk's sncRNA profile;
- SncRNA content might mediate nutrigenomic effects of milk in human nutrition.

Review

The neglected nutrigenomics of milk: What is the role of inter-species transfer of small non-coding RNA?

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Running title: Nutrigenomics of dairy products: Focus on short non-coding RNA

ABSTRACT

The characterization of small non-coding RNA (sncRNA) in food has become part of the field as a promising field of nutrigenomics. Milk contains sncRNA that are protected by extracellular vesicles which makes them resistant to digestive processes and possibly absorbable by the human gut. Due to the high conservation of sncRNA, these molecules might mediate inter-species gene expression regulations, opening numerous applications in the field of human nutrition. These include the modulation of sncRNA milk profile through diet, both in humans and dairy animals, livestock rearing methods, food technology, but also the production of infant formulas or the usage of sncRNA as biomarkers. SncRNA contained in milk might contribute to the elucidation of the long-term effects of milk consumption in the human diet, confirming the application of nutrigenomics in both health promotion and food production areas. The main aim of this mini-review is to introduce this aspect of nutrigenomics illustrating both promising aspects and pitfalls.

Keywords: nutrigenomics; epigenetics; milk; small non-coding RNA; nutrition; microbiome.

List of abbreviations

DNMT	DNA methyl transferase
EV	extracellular vesicles
miRNA	micro RNA
mRNA	messenger RNA
piRNA	PIWI-interacting RNA
siRNA	short interfering RNA

sncRNA small non-coding RNA

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1. Small non-coding RNA and gene regulation

Small non-coding RNA (sncRNA) are untranslated transcripts (~21–34 nucleotides long) that regulate 40 % to 60 % of gene expression in humans. Several types of sncRNA have been described. MicroRNA (miRNA), endogenous short interfering RNA (endo-siRNA) and Piwi-interacting RNA (piRNA) are the most extensively studied. They differ in biogenesis, length, and mechanisms through which they accomplish their biological functions (Carvalho Barbosa, Calhoun & Wieden, 2020). The ability to downregulate gene expression in the cytoplasm by pairing with target mRNA, mediated by the assembly of an effector complex, i.e., RNA-induced silencing complex (RISC) (Bartel, 2004; Fabian & Sonenberg, 2012), is a

function of sncRNA (miRNA in particular), but it is not the only one. They can also act inside the nucleus (Sarshad et al., 2018), where they can post-transcriptionally regulate small and long non-coding RNA or even promote gene expression at the transcriptional level through mechanisms that have not been completely elucidated (Liu et al., 2018). Moreover, the specific subclass of piRNA can suppress the activities of transposable elements, regulate chromatin architecture to control genomic stability and modulate stability and translation of messenger RNA (Jodar & Anton, 2018). SncRNA have been found not only in the intracellular environment (as initially hypothesized) but also in biological fluids. Extracellular vesicles (EV), which are released as a means of intercellular communication, and fat globules have been shown to protect and carry sncRNA (van Herwijnen et al., 2018; Wolf, Baier & Zempleni, 2015), thus facilitating the long-range intercellular effects of these molecules (Ferrero et al., 2017; Turchinovich, Samatov, Tonevitsky & Burwinkel, 2013; Yeri et al., 2017). However, the presence of sncRNA in biological fluids is not exclusively associated with EV (Turchinovich, Weiz, Langheinz & Burwinkel, 2011). Biological fluids that contain sncRNA include blood, urine, saliva, cerebrospinal fluid, sperm, tears and milk (Ferrero et al., 2017; Izumi et al., 2012; Park et al., 2009; Weber et al., 2010; Yeri et al., 2017). Their unexpected resistance to degradation in the extracellular environment led to sncRNA being considered strong biomarker of health and disease, as well as a promising prognostic tools. Alterations in sncRNA profile has been shown in numerous pathological conditions, not only in the measurement of tissue-specific expression patterns (de Almeida, Fraczek, Parker, Delneri & O'Keefe, 2016; Lekka & Hall, 2018), but also in the detection of peripheral sncRNA in body fluids (Gupta, Bang & Thum, 2010; Y. Jin et al., 2019; Mi, Zhang, Zhang & Huang, 2013; Redell, Moore, Ward, Hergenroeder & Dash, 2010; Roth et al., 2010; Santamaria-Martos et al., 2019).

2. *Inter-species gene expression regulation through small non-coding RNA: Any nutrigenomic effect?*

Nutrigenomics studies how food can modulate gene expression (Bordoni & Gabbianelli, 2019). This discipline focuses on the role of macro- and micro-nutrients, bioactive compounds and dietary regimens in regulating gene expression and consequentially affecting the health status. In particular, nutri-epigenomics investigates the role of epigenetics in mediating the effects of food on gene expression. The term nutrimiromics has been coined to define the study of how nutrients and bioactive molecules (e.g., selenium, zinc, resveratrol, curcumin and quercetin) can modulate miRNA concentrations in the human body (Quintanilha, Reis, Duarte, Cozzolino & Rogero, 2017). While the ability of food to modulate endogenous sncRNA production has been extensively shown, the existence of food-derived sncRNA, that remain stable and can potentially be absorbed, was shown by Yang, Hirschi and Farmer in 2015 and it is still a discussed topic (Yang, Hirschi & Farmer, 2015).

Exogenous sncRNA have been found in both plant- and animal-derived foods. The mobility of sncRNA from one species to another is considered one of the main mechanisms for cross-talk between different organisms, even between species from different kingdoms (Choi, Um, Cho & Lee, 2017; Zeng et al., 2019). While the sequence of some miRNA is specific to a few plants or animal lineages, others are conserved in animals and plants (Ha, Pang, Agarwal & Chen, 2008; van Herwijnen et al., 2018). Since foods contain sncRNA that could potentially target human genes, it has been speculated whether an inter-species genomic regulation by sncRNA could exist (Li, Xu & Li, 2018; Liang et al., 2012; Zempleni, Baier, Howard & Cui, 2015; Zhang, Chen, Yin, Zhang & Zhang, 2019; Zhao, Cong & Lukiw, 2018) and have a specific role in disease pathogenesis (Perge, Nagy, Decmann, Igaz & Igaz, 2017). However, although sequence conservation of miRNA and target genes may suggest conservation of expression patterns and functions, several questions remain to be addressed: the stability and

bioavailability of sncRNA as a function of the food matrix, the efficiency of their uptake in the gut system and the amount of xeno-microRNA needed for biological actions. Major concerns about the possibility that sncRNA could have significant biological effects in mammals have been raised (Dickinson et al., 2013; Snow, Hale, Isaacs, Baggish & Chan, 2013; Witwer, McAlexander, Queen & Adams, 2013). On the other hand, some authors have described a significant bioavailability of both plants and animal-derived sncRNA (Baier, Nguyen, Xie, Wood & Zempleni, 2014; Benmoussa et al., 2020; Manca et al., 2018; Wu et al., 2019), suggesting that they might be absorbed in the intestine and transferred into the blood circulation (Liang et al., 2014; Liang et al., 2015; Yang, Farmer, Agyekum, Elbaz-Younes & Hirschi, 2015; Yang, Farmer, Agyekum & Hirschi, 2015; Zhao et al., 2018). Izumi et al. (2015) showed that miRNA from milk might be taken up by human intestinal cells (Liao, Du, Li & Lönnerdal, 2017) and macrophages (Izumi et al., 2015; Lässer et al., 2011). This suggested that certain types of food, beyond being a source of macro- or micro-nutrients, bioactive molecules, and energy, might also provide biologically active sncRNA. Although the possibility of systemic effects is still open (see paragraph 4 for more details), the exposure to exogenous sncRNA coming from food has been reported (Ledda, Ottaggio, Izzotti, Sukkar & Miele, 2020; Li et al., 2018; Sanchita, Trivedi, Asif & Trivedi, 2018; Vaucheret & Chupeau, 2012), and the possibility that they might exert significant biological effects in mammals needs further study (Asgari, 2017; Nguyen, 2020).

Bacteria could also produce miRNA-like molecules that could modulate the host's gene expression, as previously shown for sncRNA produced by viruses (Cardin & Borchert, 2017; Duval, Cossart & Lebreton, 2017; Kincaid & Sullivan, 2012; Shmaryahu, Carrasco & Valenzuela, 2014). However, only limited data is available on their ability to target human gene expression (Choi et al., 2017; Lee, 2019). On the other hand, bacteria manipulate the expression of various miRNA in the host to modulate cellular processes that favors their

166 survival and proliferation (Ahmed, Zheng & Liu, 2016; Duval et al., 2017). Moreover, it has
167 been shown that fecal miRNA (including those deriving from food) can shape the gut
168 microbiota, thus representing a potential future strategy for manipulating the human
169 microbiome (Liu et al., 2016).

170

171 ### *3. sncRNA in milk*

172 Milk is one of the most important biological fluids, rich in macro- and micro-nutrients but
173 also bioactive compounds, like antimicrobial molecules, growth factors, immune cells and
174 antibodies. Moreover, milk is a rich source of all types of sncRNA (Martin, 2017; Testroet et
175 al., 2018; Weber et al., 2010), which are stable with degradative conditions (Izumi et al.,
176 2012; Zempleni et al., 2016) and *in vitro* digestion (Benmoussa et al., 2016; Rani, Vashisht,
177 et al., 2017). Their stability is favored by the presence in the emulsion of EV that protect
178 sncRNA from enzymatic degradation and facilitates their uptake by endocytosis (Baier et al.,
179 2014; Pathan et al., 2019; Tomé-Carneiro et al., 2018; Zhou et al., 2012). Probably for this
180 reason, milk is the biological fluid that contains the highest level of sncRNA relative to its
181 volume, and these sncRNA are stable with acidic conditions, resistant to RNAse and to
182 degradation with freeze-thaw cycles (Golan-Gerstl et al., 2017; Pieters et al., 2015; Rani,
183 Yenuganti, Shandilya, Onteru & Singh, 2017; Weber et al., 2010).

184 Despite numerous inter-species differences on sncRNA profile have been measured, analysis
185 of miRNA in milk from different species showed that some microRNA are persistently
186 abundant and overlap between human and other mammal's milk. Benmoussa and Provost
187 (2019) have provided a complete overview of the miRNA characterized in previous studies
188 and identified the top 10 microRNA found in human, cow or goat milks. The existence of
189 these recurrent "milk miRNA" suggests a conserved evolutionary process that leads to the

190 release of specific microRNA in milk, maybe because of potentially conserved functions in
191 lactation and, possibly, for the newborn's development and health (Kosaka, Izumi, Sekine &
192 Ochiya, 2010; Stephen et al., 2020; van Herwijnen et al., 2018; Zempleni et al., 2016). It has
193 also been speculated that milk-derived sncRNA may be involved in the "epigenetic priming"
194 of the newborn (Perge et al., 2017). Since the digestive tract of infants is far less developed
195 and has less harsh conditions (lower acidity and lower enzymatic activity), it allows immune
196 cells and, other cells in milk, to survive and settle within the infant's digestive tract wall (Le
197 Huërou-Luron, Blat & Boudry, 2010; Mirza et al., 2019). Thus, it has been hypothesized that
198 these specific conditions might led to the transfer of dietary microRNA through milk EV to
199 infants (Izumi et al., 2012; Kosaka et al., 2010; Zhou et al., 2012), having a role in regulating
200 their development. Carney et al. (2017) showed that the miRNA profile of breast milk from
201 mothers of premature infants differs from that of mothers of term infants, suggesting that
202 premature delivery might stimulate the secretion of a milk with a microRNA profile that may
203 have adaptive functions for growth in premature infants (Carney et al., 2017).

204 The discovery of sncRNA in milk has raised the question about what is the function of these
205 regulatory elements in this biological fluid. Numerous miRNA released in milk originate
206 from epithelial cells (mammary gland cells), but the identification of abundant immunity-
207 related microRNA suggested that they can also be released in milk from immune cells. The
208 presence of miRNA in colostrum (Van Hese, Goossens, Vandaele & Opsomer, 2020)
209 suggested a potential role of miRNA as important regulators of both immune- and
210 development-related processes (Alsaweed, Lai, Hartmann, Geddes & Kakulas, 2016a; Carney
211 et al., 2017; Kosaka et al., 2010; Q. Zhou et al., 2012). MiRNA can regulate B- and T-cell
212 differentiation and affect interleukin production of macrophages, and their role in modulating
213 inflammation has been documented (Rebane & Akdis, 2013). Bovine milk EV and associated
214 miRNA have been shown to be bioavailable and to distribute among murine tissues,

accumulating in particular in the liver and, to a lesser extent, in the spleen of mice (Manca et al., 2018), supporting the possibility of systemic effects induced by exogenous sncRNA.

Among milk miRNA, miR-148a is one of the best studied. Its sequence is highly conserved (it is identical between humans and cows), and it regulates DNMT1 and DNMT3 expression, thus affecting epigenetic homeostasis of DNA methylation. MiR-148a has been shown to have a role in the molecular mechanisms of oncogenesis (Li, Deng, Zeng & Peng, 2016). Both positive and negative associations with cancer have been proposed for miR-148a: some authors addressed it as a risk factor (Melnik & Schmitz, 2019), while others suggested that it might exert a protective effect against cancer in infants (Golan-Gerstl et al., 2017). MiR-148a may regulate food intake and adipogenesis (Melnik & Schmitz, 2017) and it could also affect the development of the nervous system (Li et al., 2016). Another miRNA highly expressed (particularly in cow's milk) and having biologically relevant functions is miR-21-5p. This miRNA regulates cell growth and proliferation (Kumarswamy, Volkmann & Thum, 2011), and it has been defined as an oncomiR (Feng & Tsao, 2016). Another miRNA typical of cow's milk and with high homology to the human sequence is miR-30d. This miRNA targets the 3′-UTR of TP53 (an oncosuppressor gene) to down-regulate the tumor suppressor p53 protein levels, thus bringing into question the potential beneficial effects of milk in the long-term (Melnik, 2017; Melnik & Schmitz, 2019). These are just a few examples aimed to focus on the heterogeneous sncRNA profile in milk, which is rich in miRNA that can have both positive and negative effects on health (Svoronos, Engelman & Slack, 2016). MiRNA deregulation is typically found in cancer, with oncomiRs that are overexpressed, while tumor suppressive miRNA are underexpressed in cancer cells. If it is confirmed that milk miRNA can enter both normal and tumor cells and affect their biological functions (Golan-Gerstl et al., 2017), studies on sncRNA profiling might help to determine additional molecular mechanisms through which potentially harmful effects of milk consumption might be

mediated. Considering that the long term safety of milk consumption is still debated due to its potential implication on cancer promotion (Fraser et al., 2020; Jeyaraman et al., 2019; Lu et al., 2016). Therefore, investigating the role of exogenous sncRNA might give further insights (Pirim & Dogan, 2020). To better understand which pathways could be modulated by miRNA contained in milk, several authors (Benmoussa & Provost, 2019; Golan-Gerstl et al., 2017) extensively reviewed the implications for human health of the most conserved miRNA in milk, considering also their bioavailability and bioaccessibility.

While an extensive characterization of the milk miRNA profile has been defined, little is known about which endogenous siRNA and piRNA are present in milk. Considering that endogenous siRNA and piRNA not only modulate gene expression but also affect genome stability, more research is needed. This topic also warrants attention considering that EV have been explored as nanodevices for the development of new therapeutic applications, and milk EV may be viable natural nano-carriers for the delivery of miRNA- and siRNA-based drugs (Aqil et al., 2019; Arntz et al., 2015). Since the interest towards these technologies is increasing (Chakraborty, Sharma, Sharma, Doss & Lee, 2017; Galley & Besner, 2020; Gorji-Bahri, Hashemi & Moghimi, 2018), their application in different therapies might also benefit from more research.

4. Concerns on the effects of exogenous sncRNA exposure

Important discrepancies on the biological effects of these exogenous sncRNA sources in humans have been identified in the scientific literature (Li et al., 2018; Zhang et al., 2019). A large part of the scientific community recommends caution in drawing definite conclusions because of potential fallacies. Issues include contamination, technical artifacts and confirmation bias (Fromm, Tosar, Lu, Halushka & Witwer, 2018; Heintz-Buschart et al.,

2018; Kang et al., 2017; Witwer, 2018; Yang, Hirschi, et al., 2015). Different methods and their specific limitations have probably led to the discrepancies. The sensitivity of the methods and the proper use of internal and external controls may also affect the reliability of the results. The role of confounders in plasma miRNA analysis has also been raised by Wang et al. (2018). The existence of significant similarities between bovine and human miRNA sequences is believed to be responsible for false positive results in the detection of dietary miRNA in human blood. Indeed, the existence of systemic effects of sncRNA is still argued. Important issues in replicating the evidence to confirm the presence of high levels of circulating miRNA in blood after milk consumption have been raised (Auerbach, Vyas, Li, Halushka & Witwer, 2016). While some studies reported that exogenous miRNA are active in the recipient organisms (Zhang et al., 2012), and regulate gene expression at distant organ sites, subsequent studies have been unable to confirm an active role of diet-derived miRNA in mammalian circulation or tissues (Auerbach et al., 2016; Kirchner, Buschmann, Paul & Pfaffl, 2020; Title, Denzler & Stoffel, 2015; Title, Denzler & Stoffel, 2015; Witwer, 2014; Witwer & Zhang, 2017; Zempleni, Baier & Hirschi, 2015). The hypothesis that a weaker or absent gut barrier (i.e., at early stages of development or in gut disorders) could facilitate the passage of RNA molecules has been recently investigated in animal models. Kirchner et al. (2020) confirmed that a transfer of protein from maternal milk to the child's circulation exists before gut closure, but they were not able to show an increased transfer of RNA molecules with the same conditions in calves. An increased transfer of RNA was not measured in the presence of increased gut permeability in mice (Yang, Elbaz-Younes, Primo, Murungi & Hirschi, 2018).

The hypothesis of a systemic role of milk sncRNA in systemic circulation remains to be studied (Fritz et al., 2016; Wang et al., 2018). However, their presence in food and consequentially in the gut, should be considered for the potential local effects. Indeed, food

derived miRNA have been detected in feces and gastrointestinal mucosa (Link et al., 2019). Since miRNA play a major role in determining intestinal cell fate (Dalmasso et al., 2010), their uptake from colonocytes (Liao et al., 2017) and macrophages (Lässer et al., 2011) might exert significant effects on gut and intestinal immune systems that needs further investigations. For example, it has been shown that milk exosome and miRNA depletion exacerbates cecal inflammation in an animal model (Wu et al., 2019).

A substantial portion of EV in milk seems to escape absorption and enter the large intestine, and given the previously described interplay between host and bacteria by sncRNA, it is likely that sncRNA contained in milk may also modulate the human microbiome. Indeed, plant-derived exosomal microRNA have been shown to modulate the microbiome (Teng et al., 2018), and alterations of the gut microbiota were measured after oral administration of bovine milk-derived EV in mice, whose intestinal immunity was enhanced by the treatment (Tong et al., 2020). The ability of miRNA to modulate the microbiome has been shown for miRNA contained in feces independently of their origin. Oral administration of synthetic miRNA affects specific bacteria in the gut (Liu et al., 2016). Similarly, plant-derived exosome-like nanoparticles that contain RNA were taken up by the gut microbiota, whose composition was altered (Huang, Pham, Davis, Yu & Wang, 2020; Teng et al., 2018; Zhou, Paz, Sadri, Fernando & Zempleni, 2019). It has also been shown that miRNA in human milk differed between mothers supplemented with probiotics instead of placebo (Simpson et al., 2015). These results suggested that sncRNA contained in food may be used to manipulate the microbiome.

The possibility of modulating the sncRNA profile contained in food (including milk) opens the way to interesting future prospectives for the development of functional foods that might be active because of their optimized sncRNA content.

5. *Implications of milk sncRNA intake for human nutrition and future prospectives*

5.1 Modulating the sncRNA profile: From diet to food technology

SncRNA expression pattern in milk is influenced by several factors, such as maternal nutrition and environmental exposures (Chen et al., 2017; Wang et al., 2016). The fat content of the maternal diet appears to have a major effect on miRNA's expression in milk and in the neonate (Van Hese et al., 2020). Since plant miRNA have been detected in human breast milk (Lukasik, Brzozowska, Zielenkiewicz & Zielenkiewicz, 2017), maternal plant intake might impact breast milk's sncRNA profile. Not only diet but also pathological conditions of the mother might modulate the sncRNA profile of their milk. For example, breast milk-derived EV from mothers with type 1 diabetes show aberrant levels of miRNA (Mirza et al., 2019). Since sncRNA of maternal milk might play a role in the development of the newborn's immune system by shaping its microbiome (Le Doare, Holder, Bassett & Pannaraj, 2018), the modulation of sncRNA induced by diseases or dietary regimens during lactation should be taken into account. The immunological state of the mammary gland seems to affect miRNA expression as well. This is true in humans, where alterations of sncRNA in milk have been suggested as a biomarker of different pathologies (Ferrero et al., 2017; Kelleher et al., 2019; Rebane & Akdis, 2013), but also in cows diagnosed with subclinical mastitis (Duval et al., 2017; Sun et al., 2015), that show alterations of their milk's miRNA profile.

Since the sncRNA profile in milk is complex, to achieve an optimized composition in terms of these regulatory elements is an ambitious goal. Despite the presence of several negative effects induced by some sncRNA, the complete removal of these molecules in milk might not be the best solution (Golan-Gerstl et al., 2017; Wu et al., 2019). Ideally, the selective

337 removal/enrichment of some sncRNA in milk might be the answer, but it still represents a
338 future prospective (Gessner et al., 2019; Nguyen, 2020). Here the authors review the
339 mechanisms that, currently or in the future, could be potentially applied to optimize sncRNA
340 profile in milk (Figure 1).

341 A strategy to modulate the sncRNA profile could be the application of some processes
342 regularly used in food technology to guarantee the microbiological safety of milk. Although
343 a limited loss of sncRNA during storage has been shown (Howard et al., 2015; Izumi et al.,
344 2012), raw milk and its fat derivatives are rich in miRNA (Melnik & Schmitz, 2017). This is
345 likely due to the high stability of EV at low pH, after boiling and after multiple freeze-
346 thawing cycles (Pieters et al., 2015). Pasteurization (78°C) and homogenization of milk have
347 a minor effect on the miRNA profile (Golan-Gerstl et al., 2017). This treatment does not
348 affect the recovery of miR-148a, which has been detected at high level in pasteurized,
349 homogenized, and skim milk fractions. On the contrary, boiling (100°C) and ultra-heat
350 treatment (130°C) of milk significantly decrease the levels of milk miRNA. It has been
351 shown that EV are significantly reduced in fermented milk (Yu, Zhao, Sun & Li, 2017). A
352 reduction in miR-29b and miR-21 was measured after fermentation, suggesting that a general
353 loss of sncRNA is likely to occur after this process. This evidence suggests that fermented
354 milk products, such as yoghurt, might exert different miRNA-dependent effects on human
355 health in comparison to pasteurized milk. Only one study investigated sncRNA in cheese (Oh
356 et al., 2017), concluding that it was not possible to correlate sncRNA profile with microbial
357 communities present in the product. Replication studies in different kinds of samples are
358 warranted to clarify the role of sncRNA in cheese manufacturing. The majority of studies
359 analyzed a few candidate miRNA after different technological treatments, but did not provide
360 a complete sncRNA profiling; thus, further studies on a complete sncRNA profiling in
361 different dairy products are warranted.

Different rearing conditions of lactating animals represent other potential factors that affect the milk's sncRNA profile. A different physiological status of the dairy cattle can modulate the levels of miRNA secreted in milk. For example, the levels of lactogenic hormones (i.e., prolactin) affect cellular and extracellular miR-148a expression in bovine epithelial breast cells (Muroya et al., 2016). Increased miR-148a expression (which is associated with a decreased DNMT1's expression) is considered an important hallmark of high performance dairy cows, that may secrete more miR-148a into their milk compared to regular cows. MiRNA profile is also altered by infections, such as mastitis induced by *Staphylococcus aureus* or *Escherichia coli* pathogens (Cai et al., 2018; W. Jin et al., 2014; Sun et al., 2015). Thus, miR-142-5p and miR-223 have been suggested as biomarkers for the early detection of bacterial infections in the mammary gland. Different miRNA profiles were detected in dairy cows fed with high- and low-quality forages (Wang et al., 2016). A modulation of miRNA profile was detected in cows exposed to dietary supplementation with 5% linseed or safflower oil, suggesting that miRNA implicated in lipid metabolism are differentially regulated (Li et al., 2015). A high-fat diet during lactation was able to alter milk's miRNA profile in mice (Chen et al., 2017). The replacement of forage fiber with non-forage fiber sources in dairy cow's diets changed the expression of milk's miRNA (Quan et al., 2020). This evidence suggests that milk composition (including sncRNA profile) is responsive to dietary manipulation and to animal rearing conditions, with direct implications for dairy production. Moreover, sncRNA profiling might represent, in the future, a tool to monitor the health and physiological status of dairy livestock. Finally, considering the effects of sncRNA on bacteria strains, different sncRNA profile might impact also fermentation and dairy production.

5.2 SncRNA in children's formulas

Although breastfeeding is highly recommended, it may not always be possible. Thus, infant formulas represent an industrially produced substitute for infant consumption that attempts to mimic the nutritional composition of breast milk as closely as possible (Martin, Ling & Blackburn, 2016). Most formulas are based on cow's milk or soymilk and characterizing the levels of sncRNA expression in these products might help to improve the nutritional adequacy of these foods (Stephen et al., 2020). The assessment of miR-148a-3p, one of the most highly expressed miRNA in milk, showed significantly lower levels of this miRNA in infant formula compared to human milk (Chen et al., 2010; Golan-Gerstl et al., 2017). This preliminary data suggests that the total amount of sncRNA is depleted in infant formulas, that lack this archaic epigenetic regulatory signaling system. This might impact the early metabolic programming and the immune system development in the newborns who cannot benefit from the maternal lactation during early life. An extensive characterization of maternal milk could help to identify the most abundant sncRNA and their functions, with the future aim to optimize the profile of these regulatory molecules. However, this is an ambitious and complex challenge. SncRNA' concentration in milk might change during lactation, like other nutrients, according to the infant's needs (Carney et al., 2017; Lukasik et al., 2017). This implies that the sncRNA profiling should be performed in different periods of lactation and that supplementation should be consequentially time-dependent. Moreover, the addition of synthetic miRNA in formulas might not have the same effect in newborns as miRNA naturally present in milk. Indeed, transient transfection of chemically synthesized miRNA showed different behaviors than endogenous miRNA, suggesting that special caution must be taken (Jin et al., 2015). As suggested by Golan-Gerstl et al (2017), miRNA could be isolated from animal sources, since about 90% of miRNA found in human milk are also present in cow's and goat's milk; however, a wider characterization of the effects of sncRNA

411 in different periods of human life is required before that companies could considered the
412 possibility to use them as a supplement (Nguyen, 2020).

413

414 *5.3 Milk sncRNA as biomarkers*

415 Molecular targets that could be used to exactly measure the amount of consumed food are
416 wanted and metabolomics is a proliferative research field. The usage of sncRNA as
417 biomarkers of food intake has been suggested, but some concerns have been raised (Witwer
418 & Zhang, 2017). Firstly, a useful marker of intake reflects both the identity and the dose of
419 the source material; conversely, the sequence conservation of miRNA is incompatible with
420 discrimination of specific food sources. Moreover, there are still some uncertainties about the
421 linear correlation between miRNA abundance in the source material and their dietary
422 absorption (Yang, Hirschi, et al., 2015). These concerns actually originated from studies
423 investigating plants' miRNA, but the same doubts can be extended to animal derived
424 sncRNA. Considering that low level of miRNA are present in body fluids, a thorough
425 sequencing, with consequential high costs, would be necessary to accomplish this goal with
426 confidence. For all these reasons, additional studies and technical implementations are
427 needed to define a practical usage of sncRNA as biomarkers of food intake. Since it has been
428 shown that sncRNA profile varies depending on environmental stimuli and infections, these
429 sncRNA might help to identify unwanted environmental exposures, status of illness in cows
430 (Ma, Tong, Ibeagha-Awemu & Zhao, 2019) or diseases in women (Kelleher et al., 2019).
431 This aspect represents a stimulating future prospective for this research field both in human
432 and veterinary medicine.

433

434 *5.4 SncRNA profiles in plant-based beverages used as milk substitutes*

A growing number of people are eliminating milk from their diet because of several side effects, first among others, lactose intolerance. The usage of "plant-based milks" such as beverages based on soy, rice, oat and coconut is spreading. Since the presence of plant-derived miRNA in human breast milk has been shown, the different sncRNA profiles between animal-derived and plant-based milk might be a further aspect to be considered in human nutrition. Treatment with plant's sncRNA has been demonstrated to systemically reduce inflammation and prevent symptoms of multiple sclerosis in an experimental autoimmune encephalomyelitis (EAE) mouse model (Cavalieri et al., 2016). This suggests that exogenous sncRNA might significantly contribute to health promotion. Due to their presence in breast milk, plant-derived molecules might have an impact not only in humans that are directly fed with them but also in their progeny (Lukasik et al., 2017). Since there are no data on the stability of sncRNA in plant-based milks up to date, further investigations are necessary. Finally, since both bovine and plant infant formulae are produced (Tzifi, Grammeniatis & Papadopoulos, 2014), research on milk's sncRNA might find further applications in child nutrition, where sncRNA are likely to contribute to infant protection and development (Alsaweed, Lai, Hartmann, Geddes & Kakulas, 2016b). Measuring the effects of milk and plant based formulas (also in relation to their sncRNA content) on the gut microflora could be of particular interest.

6. Conclusions

The characterization of sncRNA in food is an emerging research field of nutrigenomics. While a certain body of evidence is available for miRNA, few investigations have been done on siRNA or piRNA in food. Since not only gene expression regulation but also genomic stability is affected by sncRNA, further studies able to provide a complete profile of sncRNA in food are necessary. Since sncRNA are resistant to digestive processes, exogenous sncRNA

460 contained in food could be absorbed by the human gut. While systemic effects are still
461 debated, it appears likely that they can affect the gut and the resident's microbiome. These
462 bioactive molecules could contribute to the impact of food on gene expression regulation and
463 their impact on human health. These gene regulation pathways represent a bridge between
464 different animal species, and between the animal and the plant kingdoms. However, a
465 scientific consensus on this topic is still missing. Publication bias (e.g., avoiding publishing
466 negative results), might contribute to these uncertainties. Clarifying the biological effects of
467 sncRNA contained in milk could provide a complete overview on the effects of milk
468 consumption in human diet, since milk is a good source of nutrients but the full safety
469 (concerning complex environmentally-based diseases) of its intake in the long term is still
470 discussed (Fraser et al., 2020; Jeyaraman et al., 2019; Lu et al., 2016). For this reason,
471 additional studies on this topic are warranted. These would help to clarify the whole picture
472 and to identify practical applications of this research field, that range from food technology,
473 to animal rearing or infant formulas production. These applications are directed towards an
474 optimized molecular nutrition, promoting the role of molecular biology (and nutrigenomics in
475 particular) beyond basic research.

476

477 **Conflicts of interest**

478 The authors declare no conflicts of interests.

479

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482

Contributions

LB wrote the manuscript, RG revised and supervised the work. All authors have approved the final article.

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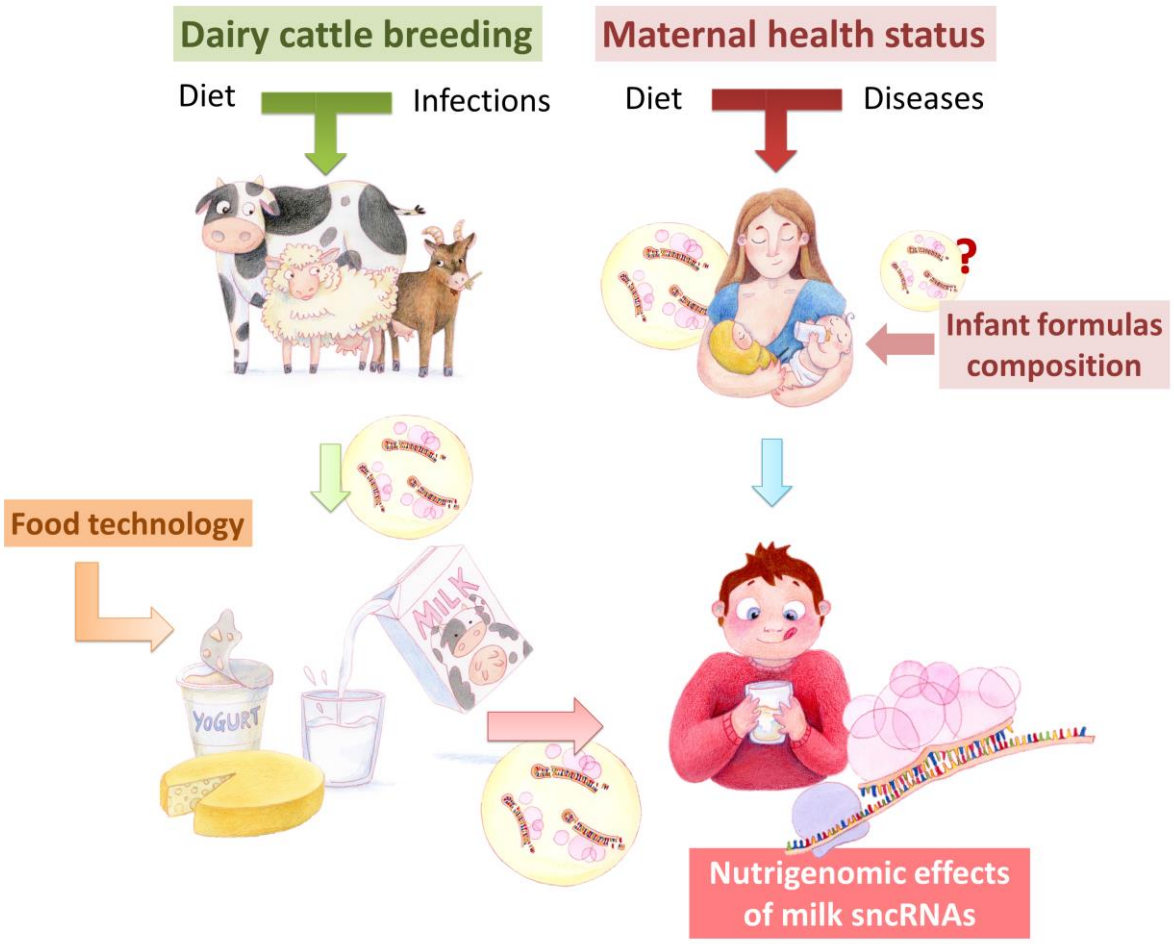
Figure legends

Figure 1. Exogenous sncRNA can be conveyed by milk and exert inter-species effects in humans. SncRNA are contained in milk produced by dairy cattle, whose rearing conditions can modulate the final profile. Different food technologies can modulate the presence of sncRNA in milk that is used in human nutrition. SncRNA are also present in maternal milk, while their content seems to be depleted in infant formulas. The exposure to different profiles of exogenous sncRNA could contribute to the heterogeneous nutrigenomic effects exerted by milk and derivatives, and consequentially impact human health. Current knowledge of possible nutrigenomic effects induced by exogenous sncRNA is represented in the bottom right of the figure (+ likely to occur; - unlikely to occur/limited evidence).

996 **Figures**

997 Figure 1.



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Competing interests

The authors declare no competing interests

Laura Bordoni, Phd

Author Agreement Statement

The authors declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.
The authors confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed.
The authors further confirm that the order of authors listed in the manuscript has been approved by all of us.
The authors understand that the Corresponding Author is the sole contact for the Editorial process. He/she is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs

Signed by all authors as follows:

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