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Article

MiR-21, MiR-148, Fatty Acid Content, and Antioxidant Properties of Raw Cow's Milk: A Pilot Study

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ABSTRACT: Intramammary gland infections can affect milk quality, with changes in composition, biochemical characteristics, and antioxidant properties. Total bacterial count (TBC) and somatic cell count (SCC) are key determinants to define the appropriate quality of raw milk and must meet specific requirements established by the European regulation. Our interest was to examine if the microbiological safety window may hide differences regarding the nutritional value of milk approved for commercialization. Therefore, we investigated microRNA (miRNA) content, fatty acid (FA) profile, and antioxidant activity in relation to SCC and TBC in microbiologically safe dairy milk. Our data show that even minor variations in SCC and TBC induced by the bacterial presence are correlated to changes in milk miRNA content, FA profile, and antioxidant properties of raw milk. This pilot study suggests that the legislative microbiological safety window can hide a range of milks, which differ for their nutritional and antioxidant values that ultimately may have effects on the consumer's health, also via epigenetic mechanisms.

KEYWORDS: *inflammation, bacterial infections, dairy milk quality, miRNAs, antioxidant activity, epigenetics*

1. INTRODUCTION

Cow's milk is a nutrient-dense food that occupies a major position in human diet thanks to its versatility and nutritional characteristics.

The nutritional quality of bovine milk is a complex parameter which results not only from biological factors (like breed, age, and sex) but also from extrinsic factors, like rearing conditions, seasonal perturbations, and animal's health. In particular, bacterial infections of the cow's mammary glands and exogenous bacterial contaminations are among the major factors that compromise the quality of milk. Contamination of milk with environmental bacteria may result from poor hygiene practices in the milking process, like inadequate cleaning of udder and teat surfaces or unsensitized milking equipment.[1] Moreover, once milk has been contaminated, bacteria may drastically increase their proliferation rate before milk reaches processing systems, if cooling of milk during transport is delayed or not appropriate. However, most of the pathogens gain access to the mammary glands by invasion through the teat canal, usually as a consequence of dirty animal beddings, and contamination of milk originating from the inside. Therefore, the total bacterial count (TBC) is a routinely used analysis to monitor the extent of bacterial contamination and to assess milk quality and safety.[2]

If pathogens break through into the mammary gland through the teat canal, it is the immunological defenses of the mammary tissue to decide whether an infection will be initiated, cleared, or not. The inflammatory response will work to destroy the invading pathogens. But, if the pathogens manage to survive, mastitis will occur, with an extent depending on the type of the causative agent and the severity of infection. The ongoing inflammation will cause pathophysiological alterations in the udder tissue that lead to changes in milk composition,[3] with an extent that reflects the degree of damage of the mammary tissue.[4−6] In the case of clinical forms of mastitis, milk is necessarily discarded because just observation reveals visible signs of alterations in the appearance of milk. On the contrary, subclinical forms of mastitis do not cause visible changes in milk and the sole observation is not sufficient to reveal an underlying inflammation. The inflammatory response induced by bacterial infections in the udder causes a rapid influx of polymorphonuclear leukocytes and the shredding of epithelial cells from the mammary gland. For this reason, the somatic cell count (SCC) can be used as a useful predictor of subclinical forms of mastitis, which are indeed characterized by increased SCC. However, also an increase in TBC can be related to mastitis, even though this parameter is not specific for the diagnosis of mastitis. In fact, an increase of TBC can also be due to the contamination of milk from exogenous bacteria during the milking process, in the case of poor hygienic conditions.

Mammary gland inflammation is associated with changes in the organoleptic properties and chemical composition of milk.[4,5] In particular, infected milk shows altered protein quality, changes in the fatty acid (FA) composition, altered lactose, ions, minerals, and antioxidant compound concentration, increased enzymatic activity, and higher pH[7−9] that ultimately affect milk quality and milk technological properties as well.[4]

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Also, an aberrant expression of small non-coding RNA molecules has been associated with mammary gland infections.[10,11] MicroRNA (miRNAs) are small non-coding molecules that have a role in the regulation of gene expression at the post-transcriptional level being responsible for mRNA degradation or impairment of mRNA translation via target base pairing.[12] Dysregulation of miRNA expression has been linked to abnormal gene expressions and regulation of immune responses to various microbial infections.[13−16] Interestingly, miRNAs are also themselves the object of regulations. In fact, bacterial infections influence miRNA expression in the host in order to perpetuate their survival and proliferation, resulting in a complex host−pathogen interaction.[17] MiRNAs are normally highly abundant in cow's milk and milk from other animal species as well.[11] Moreover, while the sequence of some miRNAs is species specific, some others are evolutionarily conserved.[18] Hence, the role of miRNAs in milk goes beyond the mere regulation of mammary gland processes and functions. Recent evidence has shown that milk-derived miRNAs may have interspecies effects and may potentially modulate the immune system or metabolic processes of milk recipients.[19] In fact, miRNAs are stable and resistant to RNase digestion, acidic environments, incubation at room temperature, or multiple freeze/thaw cycles; thus, they may reach the consumer. Furthermore, several studies have shown that miRNAs from cow's milk could be absorbed by humans and affect gene expression in peripheral blood mononuclear cells.[20,21] For all these reasons, in recent years, the interest in using miRNAs as potential disease biomarkers and milk quality control has been increasing.[22] Several studies have reported an abnormal expression of miRNAs in milk from cow diagnosed with mastitis,[23−25] most likely because of bacteria-induced inflammation. This evidence comes also from in vitro studies that confirmed the involvement of miRNAs in the inflammatory response to a bovine mastitis pathogen in mammary epithelial cells.[26]

Therefore, the monitoring of both TBC and SCC is a very important tool to assess both safety and quality of dairy milk destined to the human food chain.[27] Nevertheless, SCC and TBC threshold levels used to describe udder's health and milk hygienic quality still represent a controversial topic[28] and no consensus exists worldwide regarding their legal limits in milk for human consumption. This means that safeguard limits vary according to the countries and their policies. The regulation (EC) no 853/2004 of the European Parliament and of the Council of 29 April 2004 established that for the members of the EU, safety limits of raw milk must be SCC ≤400,000 cells/mL (rolling geometric average over a 3-month period, with at least one sample per month) and TBC ≤100,000 cfu/mL (cfu, colony forming units, rolling geometric average over a 2-month period, with at least two samples per month). Moreover, the definition of milk quality is quite complex and to give an absolute definition is not possible. Indeed, as stated by the ISO 9000 (1994) definition, for a given product, high quality is achieved when, established a set of requirements, the inherent characteristics of the product meet all requirements.[29] Therefore, based on the legislative regulations, production conditions, and market demand, the choice of parameters to assess milk quality and their threshold values may substantially vary.

We were interested to answer the following question: "do the broad microbiological safety ranges established by law, hide products with a different nutritional value?", or in other words: "are there any nutritional differences between milk that is closer to the roof or to the floor of the TBC/SCC ranges?". To answer this, we investigated if low-grade inflammatory processes or minor bacterial presence are able to affect milk FA profile, miRNA content, and antioxidant activity, ultimately decreasing the nutritional value of milk that has passed the above-mentioned quality controls, thus destined to the human food chain. In this pilot study, we selected some inflammation-related miRNAs, among the most abundant found in cow milk, which are *hsa-miR-21-5p*, *hsa-miR-29b-3p*, and *hsa-miR-148a-3p*. *Hsa-miR-148a-3p* is involved in chronic inflammatory and autoimmune diseases, *hsa-miR-21-5p* regulates the expression of proinflammatory protein PDCD4 upon lipopolysaccharide (LPS) stimulation, while *hsa-miR-29b-3p* is repressed by the NF-κB pathway and can repress TNFAIP3, a negative regulator of NF-κB pathway and changes in their expression in the case of mastitis have been reported.[30−32] For the lipid profile, we investigated the levels of saturated FAs, unsaturated FAs, and their ratios. The total antioxidant activity of milk was measured with the oxygen radical absorbance capacity (ORAC) assay and was used as a measure of milk antioxidant properties.

2. MATERIALS AND METHODS

2.1. Sample Collection. Seven Italian farms participated in the study. From each farm, two kinds of samples were collected: 10 milk samples from 10 individual cows and a pooled sample consisting in the mix of the above-mentioned samples (bulk tank milk and BT milk).

All samples were collected in the same period of the year (March 2020). All animals involved in the study were in good health conditions and were not administered with antibiotics. The cows were mainly fed with corn, hay, and barley. Milk was collected in each animal from all quarters by using automatic milking systems by taking the same volume of milk from each quarter. After collection, the milk samples from individual cows were kept on ice and immediately transferred to our laboratory for analysis of FA composition, miRNA quantification, and antioxidant activity, while BT milk samples were sent to a dedicated laboratory to measure total TBC and the SCC.

2.2. Laboratory Methods. Microbiological analysis (TBC) and SCC were performed on the seven BT milk samples by the "Agenzia per i Servizi nel Settore Agroalimentare delle Marche (ASSAM)", a dedicated laboratory for the assessment of milk quality, in the Marche region.

TBC data were obtained by using the flow-cytometry assay, while SCC was measured by a fluoro-opto-electronic method (UNI EN ISO 13366-2:2007).

The company and some producers have requested to have their name withheld.

2.3. Purification of Total RNA. Total RNA, including miRNAs, was extracted from individual milk samples by using an miRNeasy serum/plasma kit (Qiagen, Hilden, Germany). 200 μL of whole milk were thawed on ice, then diluted with 5 volumes of QIAzol Lysis reagent, mixed thoroughly by vortexing, and incubated for 5 min at room temperature. Total RNA isolation was continued according to the manufacturer's instructions. To normalize the miRNA expression data, 3.5 μL of the synthetic *Caenorhabditis elegans* Ce_miR-39_1 (miRNeasy Serum/Plasma Spike-In Control, Qiagen, Hilden, Germany; 1.6×10^8 copies/μL working solution) were added to each milk sample. Next, chloroform (in equal volume to the volume of the starting sample) was added to the homogenate, which was mixed thoroughly by vortexing, incubated for 3 min at room temperature, and centrifuged (12,000*g*, 4 °C, 15 min). The resulting aqueous phase containing total RNA was collected, mixed thoroughly with 1.5 volumes of 100% ethanol, and passed through a miRNeasy column. Total RNA was eluted in 14 μL of RNAse-free water, and RNA concentrations and purity were assessed by measuring absorbance

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levels on a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Total RNA from all native samples was immediately used for retrotranscription.

2.4. Retrotranscription of Total RNA and Quantification of miRNAs by qPCR. In this pilot study, we chose to investigate three inflammation-related miRNAs, among the most abundant found in cow milk, which are *hsa-miR-21-5p*, *hsa-miR-29b-3p*, and *hsa-miR-148a-3p*. Complementary DNA was generated using an miScript II RT with a miScript HiSpech Buffer (Qiagen, Hilden, Germany, Catalog no. 218161), according to manufacturer's instructions. Briefly, 100 ng of total RNA were polyadenylated using poly(A) polymerase, and cDNA was generated with reverse transcriptase using tagged oligo-dT primers. For reactions to occur, samples were incubated at 37 °C for 60 min, then at 95 °C for 5 min, using a standard thermal cycler. The cDNA was diluted in nuclease-free water to 1 ng/μL and 1 ng cDNA was used for the determination of miRNA expression profiles by qPCR. qPCR was performed with a miScriptSYBR Green PCR kit (Qiagen, Hilden, Germany, catalog no. 218073) on a CFX96 Real-Time PCR System (BioRad, Hercules, California, USA). The assays to detect *hsa-miR-148a-3pa*, *hsa-miR-29b-3pb*, and *hsa-miR-21-5p* were purchased from Qiagen (218300 miScript Primer Assay, Qiagen, Hilden, Germany): *hsa-hsa-miR-148a-3pa-3p*, catalogue no.—MS00003556; *hsa-miR-21-5p*, catalogue no.—MS00009079; and *hsa-miR-29b-3p*, catalogue no.—MS00006566.

The amplification procedure consisted of an initial hold at 95 °C for 15 min to allow HotStarTaq DNA polymerase activation, followed by 40 cycles made up of 94 °C for 15 s, 55 °C for 30 s, and 70 °C for 30 s. The ramp rate was adjusted to 1 °C/s for a CFX96 Real-Time PCR System. The relative miRNA expression on each sample was quantified according to the ΔΔCt method, after normalization with the spiked-in Ce-mir-39_1 control. The experiment was run in duplicates. Negative controls lacking cDNA were included in all experiments.

2.5. Antioxidant Activity of Milk by Oxygen Radical Absorbance Capacity Assay. The antioxidant capacity of milk was assessed by the ORAC assay according to Gillespie et al.[33] This assay measures free radical oxidation of fluorescein induced by the peroxyl radical initiator AAPH (2,2′-azobis-2-methyl-propanimidamide dihydrochloride) through fluorescence loss upon the addition of the sample, giving indirect information on the ability of the sample to quench free radicals. The area under the fluorescence decay curve (AUC) due to the change in fluorescence of probe over time is calculated in the presence and in the absence (blank) of the sample. Briefly, 150 μL fluorescein 0.08 μM dissolved in 75 mM pH 7 phosphate buffer (Sigma-Aldrich, St. Louis, Missouri, USA) and 25 μL milk native samples diluted 800 times in the same phosphate buffer were added to each well of a 96-well multi-detection plate. 25 μL, of phosphate buffer was used as a blank. The microplate was kept at room temperature for 10 min, then 25 μL of 150 mM AAPH (Cayman Chemical, Ann Arbor, Michigan, USA) were added to each well. The final volume of the assay was 200 μL. The microplate was shaken 10 s and the fluorescence kinetic was read every 1, 5 min for 90 min in a Fluostar Omega microplate reader (BMG Labtech, Ortenberg, Germany) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox; Calbiochem, San Diego, California, USA) at known concentrations (6,25, 12,5, 25, and 50 μM in phosphate buffer), a standard curve was generated by plotting Trolox standards against the average AUC. The ORAC of milk was calculated by subtracting the average AUC of the blank from the AUC of each sample and using the regression equation between Trolox equivalents and the net AUC.

2.6. FA Quantification by Gas Chromatography. To assess the FA content, a total of 35 FAs were selected and analyzed by gas chromatography. For the saturated fraction, the analyzed FAs were as follows: C4:0, C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0, C22:0, C23:0, and C24:0; while for the unsaturated fraction, the analyzed FAs were: C14:1, C15:1, C16:1, C17:1, C18:1, C18:2, C18:3n6, C18:3n3, C20:1, C20:2, C20:3n6, C20:3n3, C20:4n6, C20:5n3, C22:1n9, C22:2, C22:6n3, and C24:1n9. Lipids were extracted from milk according to the Folch method.[34] Briefly, 5 mL of milk was mixed with a 2:1 chloroform−methanol mixture (v/v) in a separating funnel to a final dilution 10-fold the volume of the sample. After shacking, two separated phases were obtained, the inner phase containing lipids in chloroform was collected and filtered through a fat-free paper into a rotavapor flask, dried under vacuum, and then re-dissolved in hexane.

FAs methyl esters (FAMEs) were obtained by heating for few minutes at 50 °C, the lipid mixture dissolved in hexane with a solution of KOH in methanol followed by addition of few mL of water. The upper hexane extract containing FAMEs was collected; 1 μL was injected in a Shimadzu A17 gas chromatography apparatus fitted with a split/injection system; and equipped with a flame-ionization detector (FID) and an OMEGAWAX 250 capillary column (30 m × 0.25 mm × 0.25 μm thickness, Supelco Inc., Pennsylvania, USA). Operating conditions included injector temperature at 250 °C, detector temperature at 270 °C, and nitrogen as the carrier gas. The programmed temperature column was held at 60 °C for 2 min after injection, followed by a stepwise increase of 2 °C/min to 100 °C and then to 240 °C by an increase of 5 °C/min. CromatoPlus was the data analysis software (Shimadzu, Kyoto, Japan) used for the quantification of peak areas. FAs were identified by comparison with retention times of commercial FA standards. FAME (Mix 37, Supelco) standards were purchased from Merck (Darmstadt, Germany).

2.7. Statistical Analysis. Statistical analysis was performed by using SPSS (IBM SPSS Statistics for Windows, version 24.0, Chicago, Illinois, USA). Data are reported as the mean ± standard deviation, unless specified otherwise. The Shapiro−Wilk test was used to check normality of data distribution. Logarithmic transformation was used to transform skewed data to approximately conform to normality for variables that showed a non-normal distribution. The *T*-test was used to compare the mean differences of the parameters of interest in the TBC groups and SCC groups. Pearson's correlation coefficient was measured to test parametric correlations between continuous variables. A p-value <0.05 was considered significant throughout the study. Confounding variables, like animal breed, lactation stage, and feed, were not considered in this study.

3. RESULTS

3.1. Somatic Cells and Total Bacterial Cells Count. All the seven BT milk samples had TBC and SCC within the limits established by the regulation CE no. 852/853/854/2004, which are $<1 \times 10^5$ cfu/mL for TBC and $<4 \times 10^5$ cells/mL for SCC. However, within the samples derived from seven farms, we observed not homogeneous values; for this reason, the mean value for TBC and SCC was used as threshold and milk samples were divided into two categories falling above or below the mean for each parameter (>TBC or <TBC and >SCC or <SCC). The mean value for TBC was 26,000 ± 11,781 cfu/mL with the lowest value being 2000 cfu/mL and the highest value being 93,000 cfu/mL. The >TBC group included BT milk from farms 1, 4, and 7 and the remaining 2, 3, 5, and 6 were included in the <TBC.

For SCC, the mean value was 179, 143 ± 22, and 566 cells/mL, with the lowest value being 93,000 cells/mL and the highest being 285,000 cells/mL. Thus, the >SCC group included BT milk from farms 1, 2, and 5 and the remaining 3, 4, 6, and 7 were included in the <SCC. The values of TBC and SCC measured on BT milk from each farm (1−7) are reported in Table 1.

3.2. Association between TBC, miRNA Levels, and Antioxidant Capacity in Milk. The levels of inflammatory miRNAs were relatively quantified in the 10 raw milk samples (from individual cows) from each farm. Among the milk miRNAs, *hsa-miR-148a-3p*, *hsa-miR-21-5p*, and *hsa-miR-29b-3p* were selected because of their high abundance in cow's milk

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Table 1. TBC and SCC Values Measured on BT Milk from Each Farm (1−7) Involved in the Study[a]

farms	total bacterial count (TBC) UFC/mL	somatic cell count (SCC) cell/mL
1	26,000	194,000
2	17,000	285,000
3	12,000	155,000
4	93,000	145,000
5	4000	207,000
6	2000	175,000
7	28,000	93,000
mean (±S.E)	26,000 ± 11,781	179,143 ± 22,566

[a]Mean value for TBC and SCC is also reported.

and because they have been linked to inflammatory processes. In the analyzed milk samples, *hsa-miR-29b-3p* levels were considered undetectable; thus, *hsa-miR-29b-3p* was excluded from the study and only *hsa-miR-148a-3p* and *hsa-miR-21-5p* were used for further analysis. A strong positive correlation between *hsa-miR-148a-3p* and *hsa-miR-21-5p* levels emerged in the analyzed samples (Pearson's $r = 0.76$; $P < 0.001$) (Figure 1C). Significant higher levels of *hsa-miR-148a-3p* and *hsa-miR-21-5p* were measured in milk with TBC > mean compared to milk with TBC < mean (median values in TBC < mean vs TBC > mean: −2.76 ± 0.89 vs −1.56 ± 1.055; $P < 0.001$ for *hsa-miR-148a-3p*; and −2.15 ± 0.79 vs −1.13 ± 0.98, $P < 0.001$ for *hsa-miR-21-5p*) (Figure 1A,B). When measuring the antioxidant capacity of milk, no significant differences between milk with TBC > mean milk and TBC < mean were detected (median values in TBC < mean vs TBC > mean: 0.33 ± 0.27 vs 0.48 ± 0.36; $P > 0.05$) (Figure S1).

3.3. Association between SCC, miRNA Levels, and Antioxidant Capacity in Milk. When looking at the SCC, results showed decreased levels of *hsa-miR-148a-3p* and *hsa-miR-21-5p* in the group of milk with SCC > mean compared to SCC < mean group, and these differences were statistically significant (median values in SCC < mean vs SCC > mean: −1.99 ± 1.12 vs −2.57 ± 1.07; $P < 0.05$ for *hsa-miR-148a-3p* and −1.43 ± 1.04 vs −2.08 ± 0.84; $P < 0.01$ for *hsa-miR-21-5p*) (Figure 2A,B).

Moreover, milk with SCC > mean displayed a significantly lower antioxidant activity relative to the SCC < mean milk group (median values in SCC < mean vs SCC > mean: 0.51 ± 0.29 vs 0.25 ± 0.27; $P < 0.001$) (Figure 2C).

3.4. FA Profile in the SCC Groups. Milk with high SCC (SCC > mean) was characterized by higher levels of butyric acid (median values in SCC < mean vs SCC > mean: −0.093 ± 1.307 vs 0.797 ± 1.216; $P < 0.01$), but at the same time lower omega-3/omega-6 (n3/n6) ratio (median values in SCC < mean vs SCC > mean: 3.913 ± 1.957 vs 2.917 ± 1.297; $P < 0.05$) (Figure 3A,B).

No significant differences were detected between the two SCC groups and SFAs ($P > 0.05$), monounsaturated FAs (MUFAs) ($P > 0.05$), or polyunsaturated FAs (PUFAs) ($P > 0.05$) (Figure S2A−C). Similarly, no significant differences were found with the unsaturated FAs (UFAs)/saturated FAs (SFAs) ($P > 0.05$) or PUFAs/SFAs ($P > 0.05$) ratios.

3.5. FA Profile in the TBC Groups. In analyzed samples, milk with higher TBC (TBC > mean) showed lower levels of total SFAs (median values in TBC < mean vs TBC > mean: 73.133 ± 4.056 vs 67.276 ± 4.638; $P < 0.001$) and of butyric acid (median values in TBC < mean vs TBC > mean: 0.715 ± 1.252 vs −0.265 ± 1.251; $P < 0.01$) (Figure 4A,B).

At the same time, milk in the TBC > mean group displayed a UFA profile characterized by increased levels of MUFAs (median values in TBC < mean vs TBC > mean: 22.167 ± 3.146 vs 26.660 ± 5.977; $P < 0.001$) and PUFAs (median values in TBC < mean vs TBC > mean: 3.662 ± 1.045 vs 4.913 ± 1.117; $P < 0.001$) (Figure 4C,D). Consequently, also the UFAs/SFAs ($P < 0.001$) and PUFAs/SFAs ($P < 0.001$) were higher in the TBC > mean group (Figure 4E,F). Lastly, no significant differences in the n3/n6 ratio between the two TBC groups were detected ($P > 0.05$) (Figure S3).

3.6. Correlation between miRNA Levels and FAs in Milk. From the study, *hsa-miR-148a-3p* showed a negative correlation with SFAs (Pearson's $r = -0,32$; $P < 0.01$) and butyric acid (Pearson's $r = -0.34$; $P < 0.01$) (Figure 5A), while was positively correlated with MUFAs (Pearson's $r = 0.33$; $P < 0.01$) and UFAs/SFAs ratio (Pearson's $r = 0.35$; $P < 0.01$) (Figure 5B). *hsa-miR-21-5p* was also negatively correlated with SFAs (Pearson's $r = -0.30$; $P < 0.05$) and butyric acid (Pearson's $r = -0.43$; $P < 0.001$) (Figure 5C), while it was positively correlated only with UFAs/SFA ratio (Pearson's $r = 0.24$; $P < 0.05$).

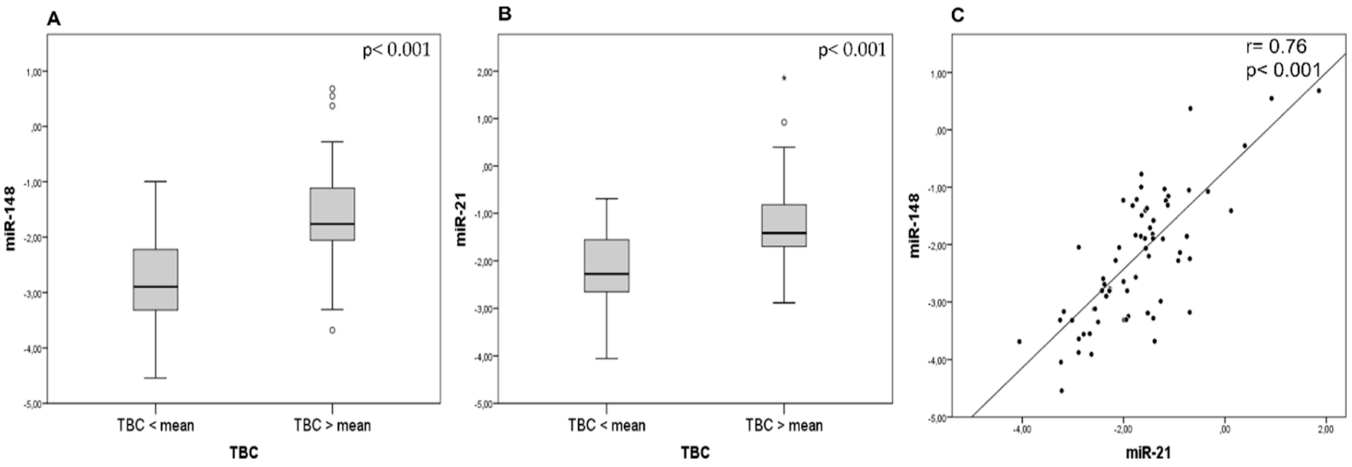

Figure 1. Relative levels of *hsa-miR-148a-3p* and *hsa-miR-21-5p* in TBC < mean and TBC > mean milk groups (A,B). Correlation between *hsa-miR-148a-3p* and *hsa-miR-21-5p* (C).

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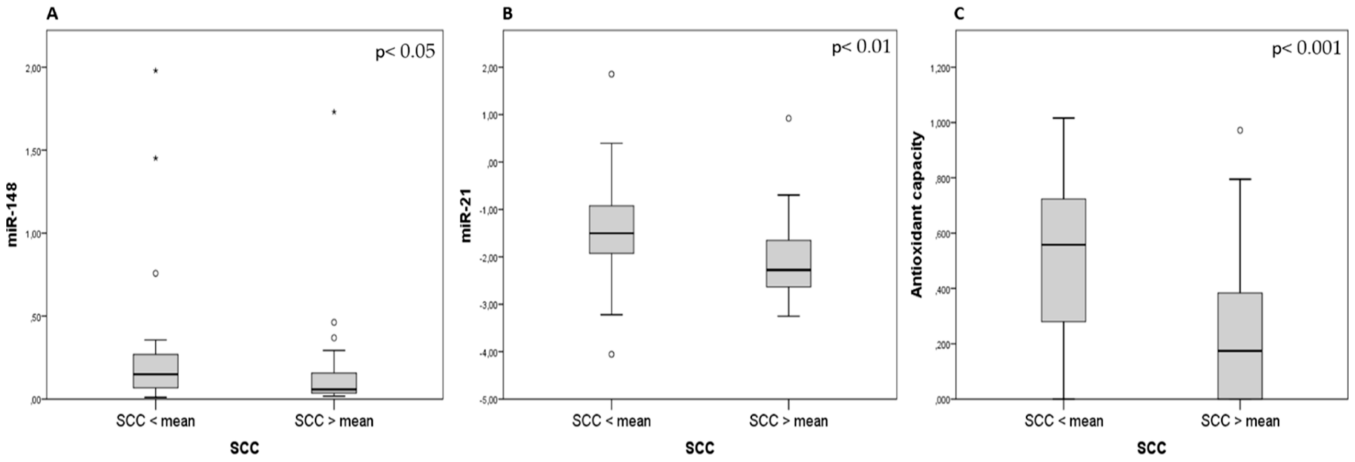

Figure 2. Relative levels of *hsa-miR-148a-3p* and *hsa-miR-21-5p* in SCC < mean and SCC > mean milk groups (A,B). Antioxidant activity of milk in the two groups (C).

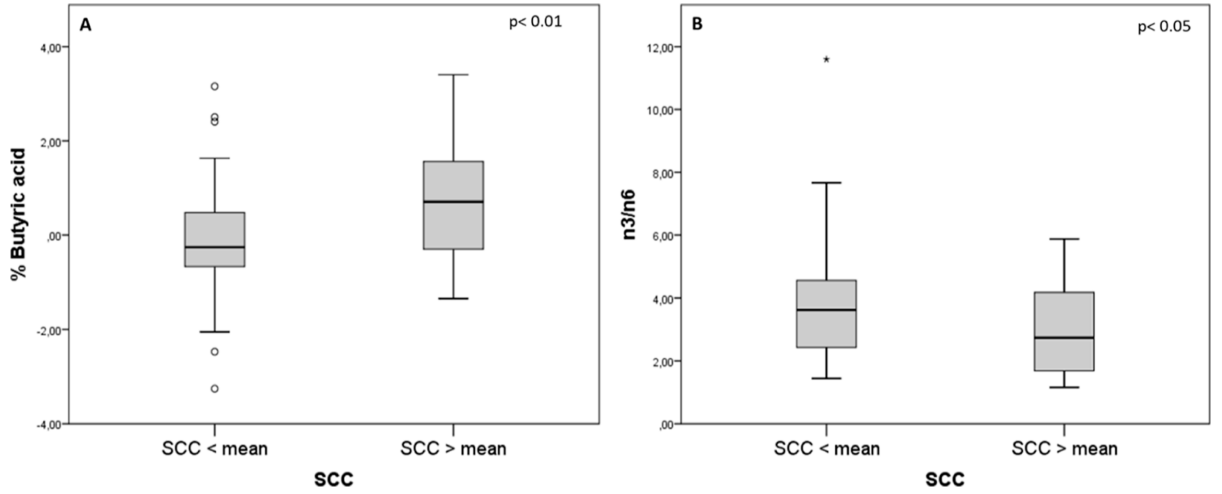

Figure 3. Relative levels of butyric acid (A) and n3/n6 ratio (B) in SCC < mean and SCC > mean milk groups.

3.7. Correlation between miRNA Levels and Antioxidant Activity of Milk. To better clarify the role of inflammatory miRNAs, their potential implication in the modulation of milk antioxidant capacity was investigated. Results showed that the antioxidant capacity of milk was not correlated to the levels of neither *hsa-miR-148a-3p* nor *hsa-miR-21-5p* (Pearson's $r = 0.17$; $P > 0.05$ for *hsa-miR-148a-3p* and Pearson's $r = 0.11$; $P > 0.05$ for *hsa-miR-21-5p*).

3.8. Correlation between n3/n6 and Butyric Acid Content of Milk. Given the beneficial effects of n3 FAs and butyric acid for human health, we investigated the relation between the n3/n6 and butyric acid content of milk and from our results, a strong inverse correlation emerged (Pearson's $r = -0.28$; $P < 0.05$) (Figure 6).

3.9. Correlation between FAs and the Antioxidant Capacity of Milk. Results showed that the antioxidant capacity of milk was positively correlated with the n3/n6 ratio (Pearson's $r = 0.25$; $P < 0.05$) and negatively correlated with butyric acid content in milk (Pearson's $r = -0.41$; $P < 0.001$) (Figure 7A,B).

4. DISCUSSION

4.1. MiRNA Levels and TBC or SCC. The presence of bacteria in raw milk is the major indicator of a poor-quality product and for that, microbiological analyses are conducted on milk soon after the milking procedure. The SCC is performed to exclude intramammary gland infections (IMIs) that do not cause visible alteration of milk (subclinical mastitis), while the TBC provides a measure of bacterial contamination from exogenous sources, as the result of poor hygiene practices during the milking process or because of improper or not immediate cooling of milk which favors bacterial proliferation.[35−38] Changes in the milk composition in the presence of IMIs result in milk with decreased quality, and they have been extensively investigated.[39−44]

Recently, milk miRNAs have been suggested as biomarkers of breast-related diseases because some of them display an aberrant profile in response to bacteria-induced inflammation of the mammary tissue, and they mainly derive from somatic cells from the mammary tissue.[9,10,23−25] The main goal of this work was to look for differences (if any) in the composition and miRNA content in milk that has passed the microbiological controls, with both SCC and TBC below the limits established by the European regulation and thus approved for commercialization. With respect to miRNA content, we investigated the levels of *hsa-miR-148a-3p*, *hsa-miR-21-5p*, and *hsa-miR-29b-3p* in raw milk that has passed all microbiological analysis (both SCC and TBC below accepted

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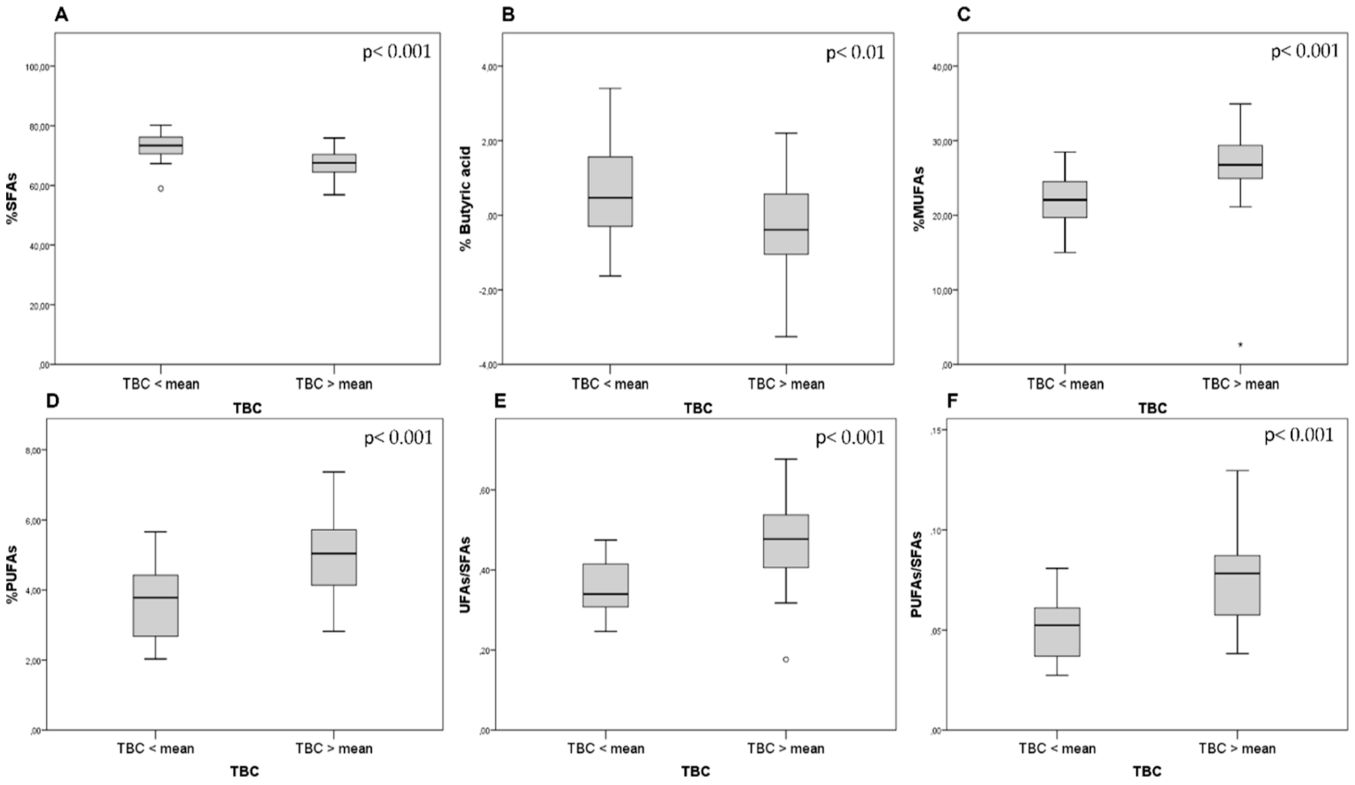

Figure 4. Percentages of SFAs (A), butyric acid (B), MUFAs (C), and PUFAs (D) in TBC < mean and TBC > mean milk groups. UFAs/SFAs (E) and PUFAs/SFAs (F) in the same milk groups are also displayed.

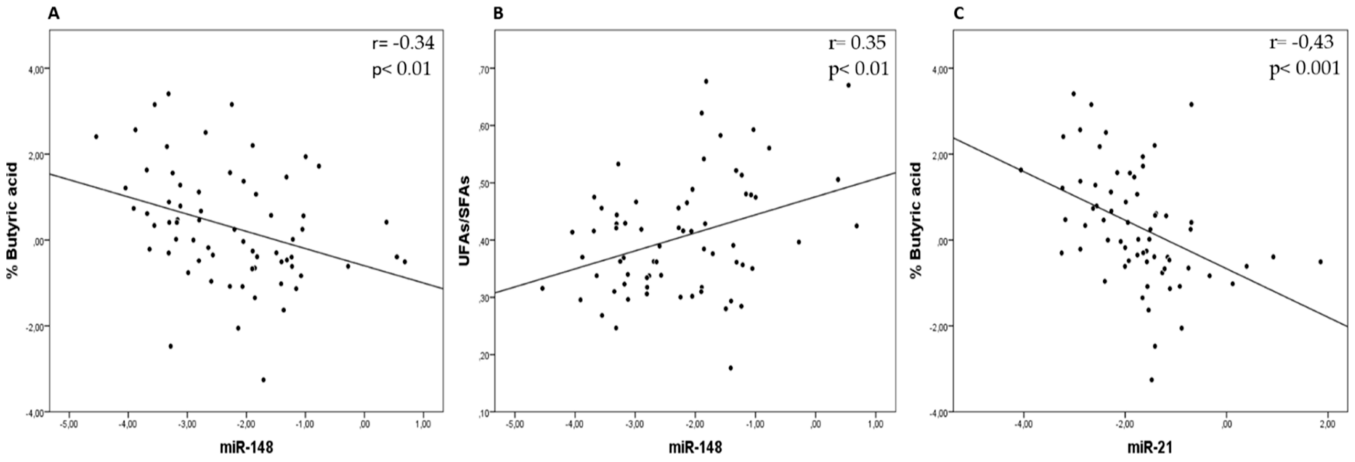

Figure 5. Correlation between *hsa-miR-148a-3p* and butyric acid (A) and UFAs/SFAs (B). Correlation between *hsa-miR-21-5p* and butyric acid (C).

limits). The levels of *hsa-miR-29b-3p* were undetectable and for this reason, *hsa-miR-29b-3p* was excluded from the analysis. Instead, *hsa-miR-21-5p* and *hsa-miR-148a-3p* were significantly overexpressed in milk with higher TBC, suggesting the existence of molecular mechanisms triggered by the bacterial presence. On the contrary, when considering the SCC, *hsa-miR-21-5p*, and *hsa-miR-148a-3p*, their levels were significantly lower in milk with higher SCC. In agreement with our findings, a recent study found that levels of *hsa-miR-148a-3pa* (and *miR-146a*) were reduced in milk from cows with mastitis compared to healthy animals[45] However, previously Lai et al., measured increased levels of *hsa-miR-21-5p* in mastitis-positive milk, compared to normal milk.[9] One hypothesis that may explain this discordance between our results and previous findings is that different pathogen strains can elicit different inflammatory responses resulting in major or minor variations of the SCC.[46,47] Unfortunately, we can just speculate it because we did not have any information on bacterial strains found in our milk samples. However, it is known that the number of somatic cells fluctuates through the course of an infection and may remain elevated (days or weeks) after bacteria are eliminated until complete healing of the gland occurs.[27,48] In this case, the lower levels of *hsa-miR-21-5p* and *hsa-miR-148a-3p* in milk with higher SCC could indicate the resolution of inflammation. In fact, even though the scenario is very complex and controversial, recent studies have attributed to *hsa-miR-21-5p* and *hsa-miR-148a-3p* anti-inflammatory roles. Dong et al. demonstrated that the overexpression of *hsa-miR-148a-3p* can

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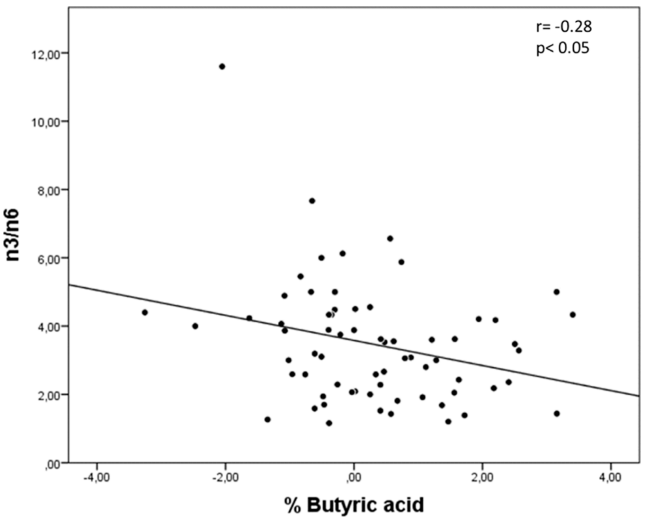

Figure 6. Correlation between butyric acid and n3/n6.

negatively regulate LPS-induced NF-κB signaling through the modulation of IL-1β in a miiuy croaker,[49] while other authors reported that *miR-21* is involved in the resolution of the inflammatory process.[32,50] Thus, *hsa-miR-21-5p* and *hsa-miR-148a-3p* increase during active inflammation in response to pathogen invasion. Indeed, our results also showed that the levels of either *hsa-miR-21-5p* or *hsa-miR-148a-3p* were upregulated in milk with higher TBC, which is consistent with the literature.

Interestingly, in our study, we found a strong positive correlation between the levels of *hsa-miR-21-5p* and *hsa-miR-148a-3p* in milk, suggesting that *hsa-miR-21-5p* and *hsa-miR-148a-3p* can work simultaneously to hinder bacterial infections. Hence, overall, our findings confirm that *hsa-miR-21-5p* and *hsa-miR-148a-3p* expressions are dysregulated in milk as a consequence of inflammatory processes in the mammary gland and that both *hsa-miR-21-5p* and *hsa-miR-148a-3p* may have an active role in the host response to pathogens. However, given conserved sequences between these miRNAs of bovine and human origin, their retrieval in milk raised some concerns over the impact of milk consumption on epigenetic regulation of the human genome.[51]

4.2. FAs, miRNAs, and SCC or TBC. FAs are important components in milk. First and foremost, milk fat is responsible for many of the sensory, physical, and manufacturing properties of milk. Second, FAs are also important components of the human diet, and milk fat has an impact on the consumer's health. Generally, in dairy cows' milk, the saturated fraction exceeds the unsaturated fraction (70% SFAs, 25% MUFAs, and 5% PUFAs).[52] However, milk fat content and FA composition are extremely variable and significantly reflect the diet of the dairy cows (fibers, energy intake, and dietary fats), even though variations in the FA composition are associated with many other factors, like genetics (breed), lactation stage, seasonal and regional effects, ruminal activity, and IMIs.[53] In particular, changes in the milk FA composition in the case of IMIs could be the consequence of the activation of the immune response of the animal, accompanied by reduced secretory activity and altered blood-milk barrier.[54] In this study, we investigated the effect of bacterial contaminations and mammary gland inflammation on the FA profile of raw milk. Our results showed that milk with increased SCC displayed a significantly higher content of butyric acid and lower n3/n6 ratio, while no significant variations in the content in SFAs, MUFAs, PUFAs, UFAs/SFAs, or PUFAs/SFAs were found. Increased levels of butyric acid in milk from cows with mastitis have been also previously found,[54−56] even though some authors reported also increased content of SFAs and medium chain FAs.[55] However, we measured higher levels of total SFAs and butyric acid in milk with lower TBC, also accompanied by lower MUFAs, PUFAs, UFAs/SFAs, and PUFAs/SFAs. These results confirm that the saturated fraction is the most represented in cow's milk under normal conditions, in the absence of significant infections.[57]

We identified a negative association between butyric acid levels and the n3/n6 ratio. Because of the positive impact of butyric acid and n3 FAs on health (may regulate the antioxidant signaling pathway),[58−60] reduced nutritional values can be attributed to milk with poor content in butyric acid and n3 or with a low n3/n6 ratio, and this is also the reason why farmers are exploring new opportunities and strategies to

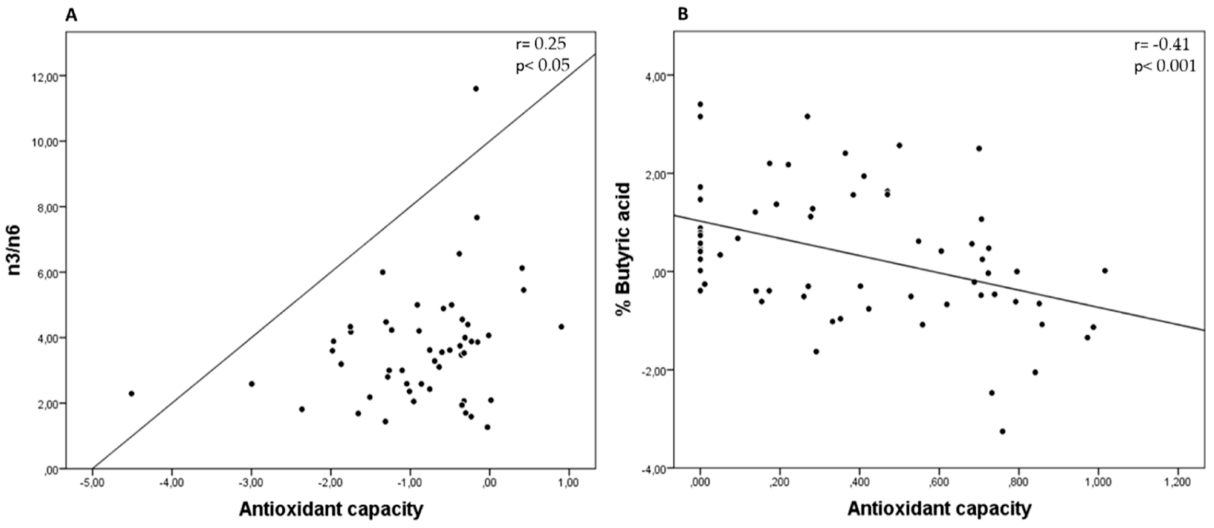

Figure 7. Correlation between the antioxidant capacity of milk and n3/n6 (A) or butyric acid (B).

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enhance the content of these beneficial FAs in milk, paying special attention to animal feeding.

FA biosynthetic processes are complex and dependent on many regulatory mechanisms, including the post-transcriptional regulation of gene expression. Previous studies have shown that miRNAs are also involved in lipid metabolism and fat deposition.[61−63] Indeed, miRNAs can regulate the translation of target mRNAs and thereby post-transcriptionally regulate adipogenesis and FA metabolism. Previously, Xia et al. demonstrated that *miR-21-3p* and *ta-miR-148a* can regulate lipid metabolism.[64] For this reason, expression levels of *hsa-miR-21-5p* and *hsa-miR-148a-3p* were correlated with the FA profile of milk in this study. The involvement of miRNAs in almost all metabolic pathways, including energy metabolism and lipid metabolism, could explain the correlation between *hsa-miR-21-5p* and *hsa-miR-148a-3p* and the FAs that emerged from our study. In particular, our results showed that the levels of either *hsa-miR-21-5p* or *hsa-miR-148a-3p* were negatively correlated to the levels of SFAs and butyric acid. On the contrary, *hsa-miR-148a-3p* has positively correlated with MUFAs and UFAs/SFA ratio, while *hsa-miR-21-5p* showed a positive association only with UFAs/SFA ratio. Several studies have investigated the relation between miRNAs and FAs in milk. For example, Du et al. found a negative correlation between the total SFA content and the levels of *miR-125a-5*,[65] while *miR-26a* was found to decrease the content of UFAs by targeting the *INSIG1* gene, in ovine mammary epithelial cells.[66] Moreover, also FAs can influence miRNA expression. For example, butyrate has been shown to upregulate the expression of *miR-10a*, *miR-24*, *miR-122*, *miR-192*, and *miR-375* in human embryonic stem cells[67] Although concerning other miRNAs and the reported studies have been conducted in vitro or on different mammalian species, these findings highlight that the relation between miRNAs and FAs can be complex and bidirectional.

4.3. Antioxidant Capacity and SCC or TBC. Antioxidant compounds are important for human health, thanks to their ability to scavenge free radicals helping in the prevention of several chronic diseases.[68] Dairy milk is a valid dietary source of antioxidants because it has its own antioxidant systems, either enzymatic (catalase, lactoperoxidase, glutathione-peroxidase, and xanthine oxidase) or not (retinoids, carotenoids, tocopherol, ascorbic acid, and polyphenols like equol).[69−71] Because previous studies have shown that the antioxidant system of milk can be altered by inflammatory processes in the mammary gland,[72] we investigated whether the antioxidant capacity of raw milk is affected by latent bacterial infections or low-grade inflammatory states, and whether FAs or miRNAs in milk have a role in its modulation. Our results are in agreement with the general scientific opinion, claiming reduced antioxidant capacity in milk in the case of IMIs.[73] In fact, although we did not measure any differences in the antioxidant capacity when considering the TBC, we found that antioxidant activity was significantly lower in milk with increased SCC. However, the total antioxidant capacity results from the contribution of all the antioxidant components, whose activity may differ in the presence of inflammation. For example, some studies found that certain enzymatic scavengers (like lactoperoxidase and glutathione peroxidase) are upregulated in milk from animals with mastitis, as a way to counteract the bacterial infection and they correlate with high SCC,[74] while antioxidant vitamins (like vitamin C, vitamin E, retinol, and carotenoids) are decreased in milk with high SCC.[72] From our results, it emerged that the antioxidant capacity of milk was positively correlated with the n3/n6 ratio and negatively correlated with butyric acid content in milk. The lower content in n3, together with a decreased antioxidant profile, may indicate a lower nutritional quality in milk with high SCC. However, high content in PUFAs is not always favorable because they are the most susceptible to peroxidation,[75,76] which is one of the main causes of chemical spoilage and changes in nutritional value, flavor, and texture of milk.[76] Moreover, the products of lipid peroxidation may represent a risk for the consumer because they may induce changes in proteins and nuclei acids.[77,78] Lastly, we measured the antioxidant capacity of milk in relation to the levels of miRNAs, but we did not find any significant correlation with the selected *hsa-miR-21-5p* and *hsa-miR-148a-3p*.

Our pilot study provides information about the effects of minor bacterial contamination in milk or latent mammary infections that are normally neglected because they do not compromise the microbiological safety of milk according to the European standards. Indeed, this safety range is quite broad and tolerates the presence of bacteria within certain limits, setting aside any differences among milk in this safety window. Even though this bacterial presence is not such as to represent a direct threat to human health (also because milk will be subsequently pasteurized), it could be linked to changes in milk miRNA content, FA profile, and antioxidant properties of milk, at least in the small sample considered in this study. Our preliminary data suggest that particular attention should be paid at the farm level to minimize the loss in the nutritional value so that a high-quality product is offered to the market. Because this work could not consider the many factors that could affect milk nutritional quality (like farming systems, management conditions, dairy breeds and age, milk yield seasons or environmental stressors, diet, or milking systems), further investigations are necessary to better clarify the impact of neglected bacterial infections on the quality of dairy milk and the possible nutrigenomics effects of milk on human health.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsfoodscitech.3c00072.

Non-significant experimental results, antioxidant activity, and relative levels, (PDF)

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Author Contributions

I.P. carried out miRNAs analysis and drafted the manuscript; D.F. carried out antioxidant and fatty acid analyses; L.B. designed research, performed statistical analysis, reviewed the manuscript; and R.G. supervised the project, reviewed, and edited the manuscript.

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■ ABBREVIATIONS

TBC, total bacterial count
SCC, somatic cell count
cfu, colony-forming unit
miRNA, microRNA
FAs, fatty acids
cDNA, complementary DNA
qPCR, quantitative polymerase chain reaction
ORAC, oxygen radical absorbance capacity
AAPH, 2,2′-azobis-2-methyl-propanimidamide, dihydrochloride
SFAs, saturated fatty acids
UFAs, unsaturated fatty acids
MUFAs, monounsaturated fatty acids
PUFAs, polyunsaturated fatty acids
n3, omega 3
n6, omega6
IMIs, intramammary gland infections
IL-1β, interleukin 1-beta
NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells
SPSS, statistical package for social science
LPS, lipopolysaccharide
FAMEs, fatty acid methyl esters

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