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Research paper

Impact of cheese whey enriched in lactobionic acid on the characteristics of fermented milks prepared with or without the probiotic *Lacticaseibacillus rhamnosus* GG

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ABSTRACT

This study explores the possibility of using the prebiotic lactobionic acid (LBA), produced through the valorization of cheese whey, a common dairy by-product, for the production of different fermented milks with/without the probiotic *Lacticaseibacillus rhamnosus* GG. The presence of LBA enhanced the growth and stability of probiotics and starter cultures, improved the antioxidant activity of the samples, and impacted the volatilome of the fermented milk during the 28 days storage at 6 °C. However, while LBA remained constant in LBA samples during both fermentation and shelf life, the acid sugar dropped below the detection limit in LBA + LGG samples, leaving higher residual lactose levels. This indicates that the probiotic prioritized LBA over the disaccharide, which could have significant implications for the final product. This work provides new insights into the use of LBA as an ingredient to functionalize fermented milk and its impact on the metabolism of lactic acid bacteria.

1. Introduction

Prebiotics include a wide range of compounds, including lactose derivatives, such as lactobionic acid (LBA), which are gaining more and more interest (Goderska, 2019a; Sáez-Orviz et al., 2022). Adebola et al. (2014) reported that the prebiotic effect of LBA is both species- and strain-dependent. Specifically, LBA was able to stimulate the growth of *Lactobacillus acidophilus* NCFM and *Limosilactobacillus reuteri* NCIMB 11951, whereas it exhibited weak or no prebiotic effect on *L. acidophilus* NCTC 1723, *L. delbrueckii* subsp. *bulgaricus* NCTC 12712, and *Levilactobacillus brevis* NCIMB 11973. The same authors demonstrated that both responsive strains were able to metabolize LBA at concentrations up to 2.5 %, while 5 % did not support growth—likely due to the lower pH levels reached under those conditions. In addition to its prebiotic properties, LBA is biodegradable, biocompatible and non-cytotoxic (Sáez-Orviz, Puertas, Marcet, Rendueles, & Díaz, 2020a). It also exhibits antioxidant, ion-chelating, and moisturizing properties (Cardoso et al., 2019) attributed to its chemical structure with multiple hydroxyl groups. Due to these properties, LBA has been proposed as an ingredient in various food formulations (e.g., yacon juice, probiotic products, ice cream) for its potential roles as a preservative, protective/prebiotic agent, and texturizer, respectively (Cardoso et al., 2019; Marques et al., 2020; Sáez-Orviz et al., 2020b; Zagorska et al., 2022). However, its potential and impact on the final food product is still being evaluated. So

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far, LBA has only been authorized by the Food and Drug Administration (FDA) for use in food in its salt form (FDA, 2017). Nevertheless, future approval of LBA by food authorities (such as EFSA) is expected (Cardoso et al., 2019; Sáez-Orviz et al., 2022). In this sense, Kiryu et al. (2009) reported the presence of LBA in the "Caspian Sea yoghurt" which was estimated to provide between 0.5 and 1.0 g of LBA annually when consumed. LBA can be produced through the chemical oxidation of lactose with bromine or as a metabolite by *Pseudomonas taetrolens* (De Giorgi et al., 2018), *Burkholderia cepacia* (Murakami et al., 2003), *Zymomonas mobilis* (Pedruzzi et al., 2011) and *Acetobacter orientalis* (Kiryu et al., 2009a). Alternatively, to minimize production costs, LBA can be produced *in situ* in dairy products (García et al., 2019; Hossain et al., 2023). Another approach that can be applied in the perspective of a more sustainable value chain is by valorizing and using the dairy by-products cheese whey as a substrate. De Giorgi et al. (2018) obtained 34.25 ± 2.86 g L^{-1} of LBA and a bioconversion yield of up to 85 ± 7.0 %, when ricotta cheese whey (scotta) was incubated with strains of *Pseudomonas taetrolens*. This study aims to develop a new dairy product with enhanced functionality by exploiting the addition of LBA, obtained through cheese whey valorization, in combination with the probiotic strain *Lacticaseibacillus rhamnosus* GG (Chr. Hansen). To assess the specific impact of each component, all possible formulations—control, prebiotic, probiotic, and symbiotic—were produced and characterized. The characterization focused on, microbial cell counts, pH, lactose/LBA concentration, antagonistic activity against food spoilage strains, antioxidant activity, and volatile compounds (GC-MS-SPME) in samples stored at 6 °C for up to 28 days.

2. Methods

2.1. Microorganisms

Pseudomonas taetrolens DSM 21104 was obtained from the DSMZ culture collection. The *Pseudomonas* sp. was grown in Tryptic Soy Broth (TSB) and maintained frozen (−80 °C) in the same growth medium supplemented with 20 % v $^{-1}$ glycerol. A commercial starter mixture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lyofast Y450 B, SACCO), in combination or not with the probiotic strain *L. rhamnosus* LGG (Chr Hansen), were used to prepare the fermented milks. Food spoilage strains *Listeria monocytogenes* Scott A, *Escherichia coli* 555, *Staphylococcus aureus* DSM 20231 were obtained from the collection of the Department of Agricultural and Food Sciences, University of Bologna.

2.2. Production of LBA by Pseudomonas taetrolens

Squacquerone cheese whey obtained from a local cheesemaker (Mambelli, Bertinoro, italy) was exploited for LBA production using the method described by De Giorgi et al. (2018). The lactose content in whey was 47.13 ± 1.419 g L^{-1} and 5 % (v v^{-1}) inoculum of an overnight preculture was used. Samples were aseptically withdrawn after 0, 7, 24, 48 and 50 h incubation and used for chromatography analysis. At the end of the process, LBA-enriched whey was centrifuged (10,000 rpm for 10 min), filtered with a 0.2 μm filter and sterilized at 121 °C for 20 min before storage at 4 °C until its use for the preparation of fermented milks.

2.3. Lactose and LBA quantification

Quantification of lactose and LBA during LBA production process and in the fermented milks stored at 6 °C up to 28 days was performed by High performane liquid Chromatography (HPLC) using the method described by (Coroli et al., 2021a) and De Giorgi et al. (2018). Both compounds were quantified according to HPLC-grade external analytical standards obtained from Sigma-Aldrich (Milan, Italy).

2.4. Preparation of fermented milk samples

The fermented milks were produced under laboratory conditions. Commercially available UHT milk was divided into 100 mL containers and each container was inoculated with 0.06 % (w v^{-1}) of lyophilized starter cultures (*L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*) (Sacco, Italy) at a concentration of 6 log cfu mL^{-1}, while *L. rhamnosus* GG was inoculated at a concentration of at least 7.5 log cfu mL^{-1}. When LBA-enriched whey was added (10 %, v v^{-1}), the final concentration of LBA in the milk was ~3.3 g L^{-1}. The inoculated fermented milk was incubated at 43 °C until it reached a pH value of 4.6, after approximately 4 h for all test conditions with no major differences between the samples in the kinetics of acidification. At the end of fermentation, the samples were stored at 6 °C for 28 days. Analyses were performed after 1, 7, 14 and 28 days of refrigerated storage. Four types of experimental fermented milks were prepared and defined as follows: Control: fermented milk containing only starter cultures; LBA: fermented milk containing starter cultures and LBA-enriched whey; LGG: fermented milk containing starter cultures and *L. rhamnosus* GG; LBA + LGG: fermented milk containing starter cultures, LBA-enriched whey and *L. rhamnosus* GG.

2.5. Strain viability in fermented milk

The viability of *S. thermophilus* and lactobacilli strains (starter cultures and probiotic adjunct) were assessed after 1, 7, 14 and 28 days of storage at 6 °C. Five grams of fermented milk were placed into 45 mL of PBS and homogenized in a stomacher (Labblender 80, Pbi International, Milan, Italy) for 3 min. Decimal dilutions of the homogenates were made in 0.9 % NaCl and 0.1 mL of appropriate dilutions was spread onto the surface of different agar media. *S. thermophilus* was counted on M17 agar (Oxoid, Basingstoke, Hampshire, UK) incubated at 42 °C, while *L. delbrueckii* subsp. *bulgaricus* and LGG were incubated on MRS with 0.05 % l-cysteine at 42 °C (aerobically) and 37 °C (anaerobically), respectively.

2.6. pH

The pH of fermented milks during 28 days of storage at 6 °C was monitored using the Basic 20 pH meter (Crison Instruments, Modena, Italy).

2.7. Antagonistic activity of fermented milks against food spoilage strains

All samples were tested for their antagonistic activity against selected food spoilage strains such as *Listeria monocytogenes* Scott A, *Escherichia coli* 555, *Staphylococcus aureus* DSM 20231 by overlay assay as described in D'Alessandro et al. (2022) with minor modifications. The pathogenic strains were routinely cultured in BHI broth at 37 °C with gentle shaking and subcultured twice before use in the experiments. Five μl of each fermented milk were spotted onto the surface of MRS plates (containing 0.05 % L-cysteine and 1.2 % agar) and incubated for 24 h under anaerobic conditions at 37 °C. Subsequently, 100 μl (corresponding to about 7–8 log cfu mL^{-1}) of the overnight subcultures of the pathogenic strains were inoculated into 10 mL BHI 0.7 % agar and poured over the spots. The plates were incubated for a further 24 h at 37 °C and then checked for the presence of a growth inhibition zone. The inhibition centers were measured from the outer edge of the spots in four directions and expressed as their average. The antagonistic activity was expressed in relation to the observed zone of inhibition: , no inhibition; +, inhibition 1–3 mm; ++, inhibition 3–6 mm; +++, inhibition >6 mm.

2.8. Antioxidant activity

The antioxidant capacity was studied by evaluating the free-radical scavenging effect using DPPH ad ABTS assays using the method proposed by Biadała and Adzahan (2021) with some modifications. 50 μL of

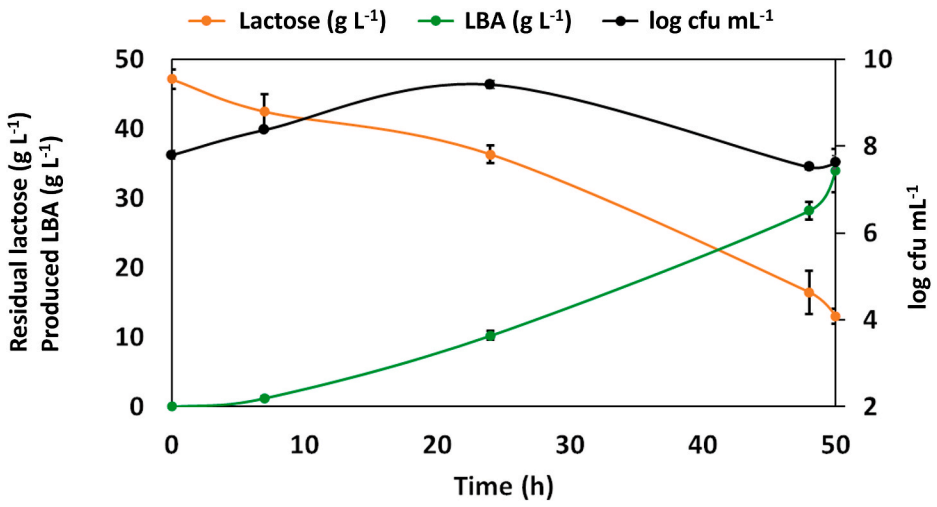

Fig. 1. Time course of LBA production (g L^{-1}) and residual lactose (g L^{-1}) during cultivation of *P. taetrolens* DMS 21104 in a 3 L bioreactor with Squacquerone cheese whey as substrate.

the supernatant of fermented milk samples, obtained by centrifugation at 12,000×*g* for 10 min, was mixed with 1.45 mL of a 0.1 mM DPPH free radical solution. After incubating the mixed solution at 25 °C for 30 min in the dark, the absorbance was recorded at 517 nm. A 95 % ethanol solution was used as blank control, and the result was expressed in terms of Trolox equivalent (mg TE mL^{-1}). The supernatant of fermented milk samples (15 μL) was mixed with 1.5 mL of ABTS working solution. After incubating the reaction mixture for 6 min at 25 °C in dark, the absorbance was recorded at 734 nm. Distilled water was used as blank control, results were expressed in terms of Trolox equivalent (mg TE mL^{-1}).

2.9. Volatile molecule profile (volatilome)

Volatile organic compounds (VOCs) of fermented milks were monitored after 1 and 28 days of refrigerated storage using gas chromatography-mass spectrometry (GC–MS) coupled with solid phase micro extraction (SPME) using the method described by D'Alessandro et al. (2023). An internal standard (4-methyl-2-pentanol, final concentration 1 mg kg^{-1}) was added to each sample before SPME analyses.

2.10. Statistical analysis

The microbiological and physico-chemical data are the means of 3 repetitions. The obtained data were analyzed by Statistica software (version 8.0; StatSoft, Tulsa, OK, USA) adopting the analysis of variance (ANOVA) and Tukey's test for data comparisons.

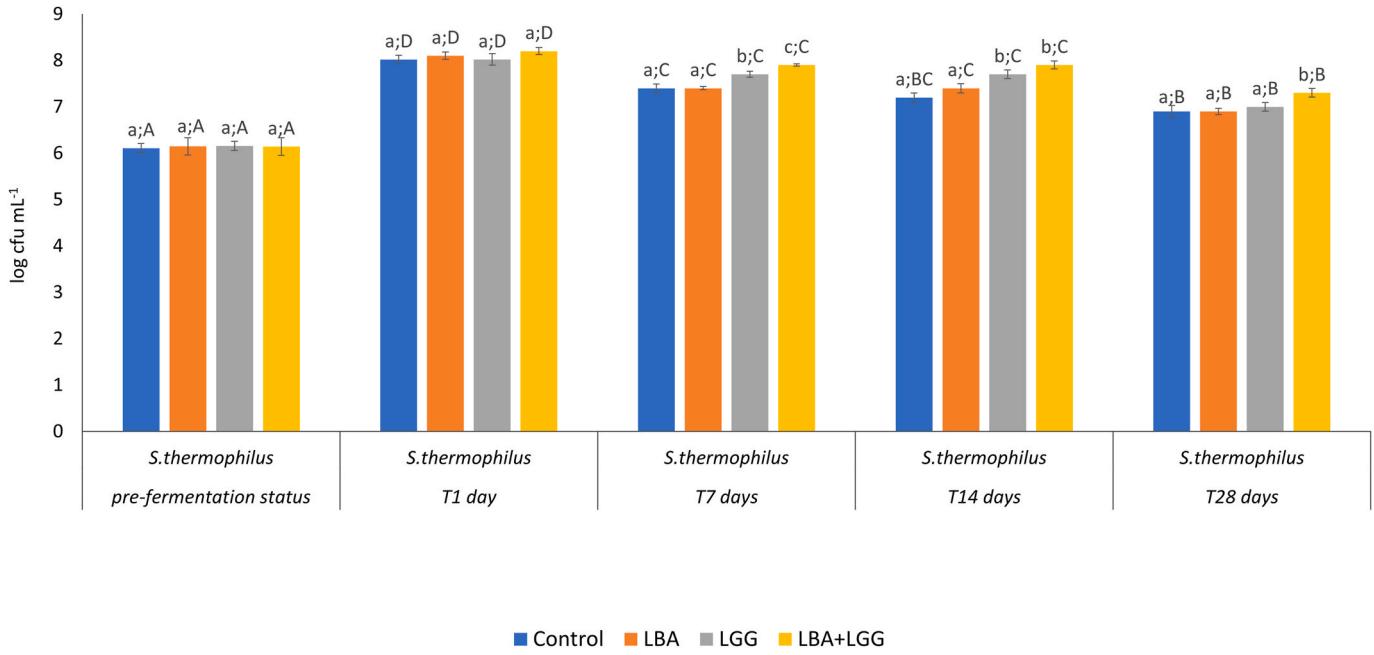

Fig. 2. Cell load viability of *S. thermophilus* (log cfu mL^{-1}) in fermented milks after the inoculum and 1, 7, 14, 28 days of storage (T1, T7, T14, T28) at 6 °C. Control: Starter cultures; LBA: Starter cultures + Lactobionic acid; LGG: Starter cultures + *L. rhamnosus* GG; LBA + LGG: Starter cultures + Lactobionic acid + *L. rhamnosus* GG. Within the same timepoint, samples with the same superscript letter must be considered not statistically different ($p > 0.05$). Different lower-case letters indicate significant differences ($p < 0.05$) between different samples with the same shelf life. Different capital letters indicate significant differences ($p < 0.05$) in the same samples during the shelf life.

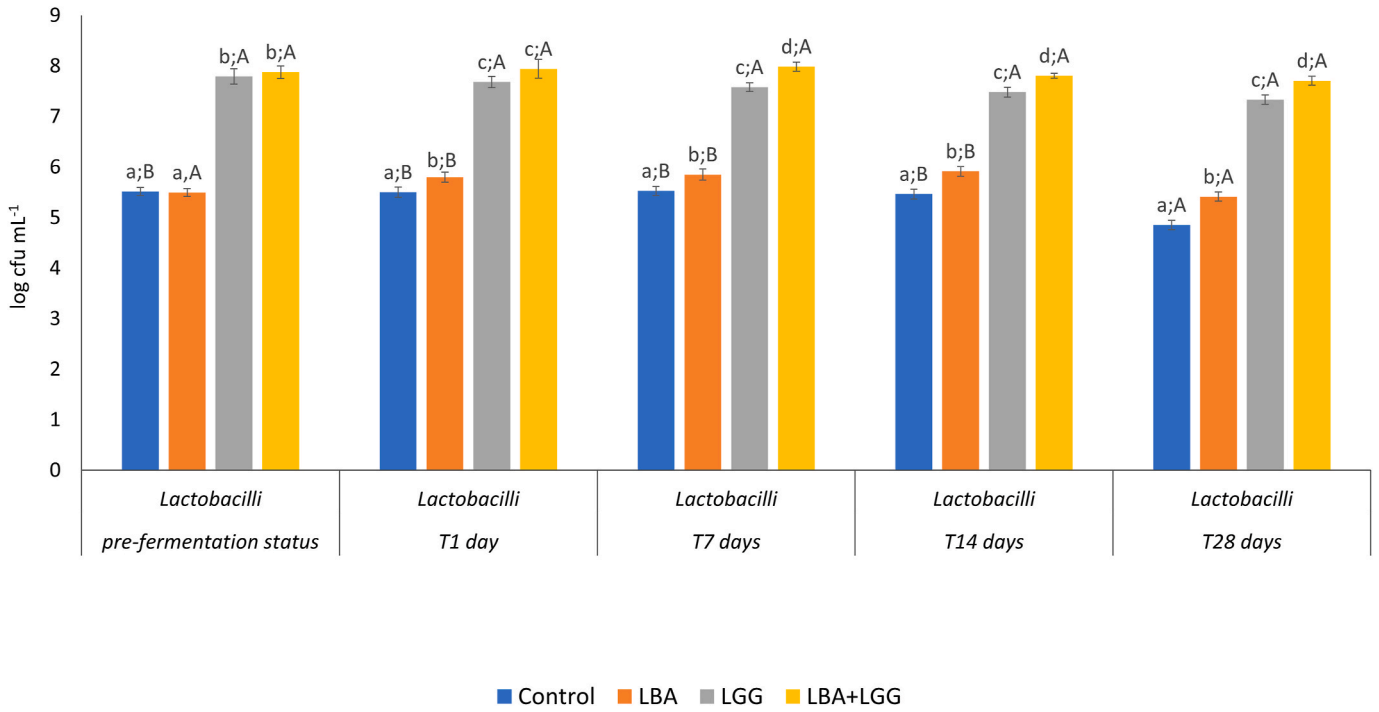

Fig. 3. Cell load of lactobacilli (log cfu mL^{-1}) in fermented milk after the inoculum and 1, 7, 14, 28 days of storage (T1, T7, T14, T28) at 6 °C. Control: Starter cultures; LBA: Starter cultures + Lactobionic acid; LGG: Starter cultures + *L. rhamnosus* GG; LBA + LGG: Starter cultures + Lactobionic acid + *L. rhamnosus* GG. Within the same timepoint, samples with the same superscript letter must be considered not statistically different ($p > 0.05$). Different lower case letters indicate significant differences ($p < 0.05$) between different samples with the same shelf life. Different capital letters indicate significant differences ($p < 0.05$) in the same samples during the shelf life.

3. Results and Sicussion

3.1. LBA production by P. taetrolens DMS 21104 from squacquerone cheese whey

For the production of LBA from squacquerone cheese whey, 5 % (v/v) of a bacterial preculture (grown for 18 h in TSB) was used as inoculum and the incubation was carried out for up to 50 h. As shown in Fig. 1, LBA production started after 7 h and increased until the end of the process. Indeed, the maximum LBA concentration of 33.914 ± 3.105 g L^{-1} (with 94.905 ± 1.659 % conversion yield) was achieved after 50 h incubation. The LBA production obtained in this study is comparable to that reported by De Giorgi et al. (2018) for ricotta cheese whey with *P. taetroleans* LMG 2336 (34.25 ± 2.86 g L^{-1} after 48 h, with 85 ± 7 % conversion yield). The sample was then centrifuged, filtered, and autoclave before its application in fermented milk formulations.

3.2. Viability of starter cultures and lactobacilli in milk and pH profile during the refrigerated storage

Figs. 2 and 3 highlight the cell counts of *S. thermophilus* and lactobacilli measured during storage at 6 °C for 28 days. These data clearly show that the cell count of *S. thermophilus* reached about 8 log cfu mL^{-1} after fermentation, with no significant differences observed between the different experimental conditions after one day of refrigerated storage. However, from the seventh day of storage, there was a decrease in *S. thermophilus* cell count, especially in the control and LBA samples, while the LGG and LBA + LGG samples showed a significantly higher cell count (7.4 vs. 7.7–7.9 log cfu mL^{-1}). On day 14, the cell counts in the LBA, LGG and LBA + LGG samples remained unchanged compared to day 7, while the control sample showed a further decrease. Finally, on day 28, a further decrease in cell count was observed in all experimental conditions, although this decrease was less marked in the LBA + LGG condition (6.9 vs. 7.3 log cfu mL^{-1}). A similar behavior regarding the viability of *S. thermophilus* was observed in other studies. According to Minervini et al. (2012), the addition of probiotic lactobacilli increased the viability of *S. thermophilus* in a Fior di Latte cheese. Also Patrignani et al. (2017) reported a higher concentration of *S. thermophilus* in fermented milk supplemented with encapsulated probiotic lactobacilli, even after 56 days of storage at 4 °C. In a more recent study, D'Alessandro et al. (2023) showed a significantly higher amount of *S. thermophilus* in a fermented soy beverage with starter cultures and vaginal probiotics compared to the same product that was not supplemented with additional cultures.

A similar trend was observed in the amount of lactobacilli detected (Fig. 3) as with *S. thermophilus*. Indeed, in the samples containing the commercial probiotic (LGG and LBA + LGG), the total load of lactobacilli was significantly higher even before fermentation (pre-fermentation), as *L. rhamnosus* GG was intentionally inoculated. Interestingly, the addition of LBA in the LBA + LGG sample had a positive effect on the cell count from 7 days onwards. In fact, the beneficial effect of LBA on the number of lactobacilli (including LGG) compared to the condition with only LGG is evident and was maintained for 28 days. Also L. *delbrueckii*, which was easily enumerated in the control and LBA samples, significantly increased in presence of the prebiotic maintaining higher values for all the storage period (≥5.8 log cfu mL^{-1}) compared to the control (≤5.5 log cfu mL^{-1}). This is particularly important considering that the number of viable bacteria during the product storage is crucial for the development of a functional product. If the survival rate is not sufficiently high, the food cannot be considered probiotic. There is no consensus on the specific number of microorganisms that need to be ingested to achieve a beneficial effect, but the estimated minimum amount is 6 log cfu per mL or g of product, and these products should be consumed in the order of 100 g every day to achieve approximately 8 log cfu of viable cells in the gut (Dinkçi et al., 2019). The fact that the LBA + LGG sample better supported the growth of the probiotic LGG is due to the expected prebiotic capabilities of LBA, which is consistent with the results of Goderska (2019b) and Sáez-Orviz et al. (2022). According to

Table 1
pH values recorded during the refrigerated shelf-life (T1, T7, T14 and T28 days) of all the investigated fermented milk products. Control: Starter cultures; LBA: Starter cultures + Lactobionic acid; LGG: Starter cultures + *L. rhamnosus* GG; LBA + LGG: Starter cultures + Lactobionic acid + *L. rhamnosus* GG. Different capital letters mean data significantly different ($p < 0.05$) for the same sample over time; different lower case letters indicate statistical differences ($p < 0.05$) among samples in a specific time point.

pH	T1 day	T7 days	T14 days	T28 days
Control	4.60 (±0.03) a; D	4.25 (±0.03) a; C	4.11 (±0.03) a; B	4.04 (±0.03) a; A
LBA	4.61 (±0.04) a; D	4.24 (±0.03) a; C	4.08 (±0.03) a; B	3.97 (±0.04) a; A
LGG	4.63 (±0.03) a; D	4.19 (±0.04) a; C	4.13 (±0.03) a; B	4.03 (±0.02) a; A
LBA + LGG	4.60 (±0.03) a; D	4.18 (±0.04) a; C	4.07 (±0.04) a; B	3.96 (±0.05) a; A

Adebola et al. (2014), the optimal prebiotic concentration of LBA was a maximum of 2.5 %, as 5 % did not promote the growth of the selected probiotics. In this sense, this work agrees well with our study, in which the addition of 0.3 % LBA supports the growth of the selected probiotic strain, maintaining also acceptable organoleptic properties.

Looking at the pH profile (Table 1), the value decreased in all the samples during the entire refrigerated period. However, the main reduction of 0.4 was observed within the first 7 days with a pH of around 4.2. In the subsequent weeks the pH dropped to around 4.1 and 4.0. It is interesting to note that at the end of the shelf life (T28), no significant differences were observed between the different experimental conditions, highlighting how the addition of LBA-enriched cheese whey at the adopted concentration (3 g L^{-1}), even when combined with LGG during storage, did not lead to excessive acidification of the samples. This could represent a positive outcome in terms of product acceptability.

3.3. Lactose and LBA concentration in fermented milks

The concentrations of lactose and LBA were determined after the inoculum of the strains (pre-fermentation: T0) as well as during the refrigerated storage (T1, T14 and T28 days), as shown in Fig. 4A and B, respectively. Hence, Fig. 4A shows that the amount of lactose decreased during the fermentation up to 35 to 20 g L^{-1} in control sample. This reduction in lactose concentration was significantly lower when LBA-enriched whey was added to the fermented milk (LBA) (around 25 g L^{-1}) and the value remained constant during the following storage period. For the same sample, LBA was not metabolized by the starter microorganisms and its concentration (3.3 ± 0.4 g L^{-1}) remained constant for 28 days (Fig. 4B). The use of the sole LGG determined a significantly higher reduction of lactose already at the end of fermentation (T1), up to 10 g L^{-1}, remaining stable till the end of the refrigerated storage. Surprisingly, the addition of LBA-enriched whey to LGG led to a reduced lactose consumption during fermentation, as the disaccharide content was not significantly different as the value at the beginning of fermentation. However, lactose progressively decreased during storage at 6 °C (Fig. 4A) suggesting a delayed microbial activity. In this sample, LBA was no longer detected already after 1 day of storage (T1) suggesting that under this experimental condition the acid sugar

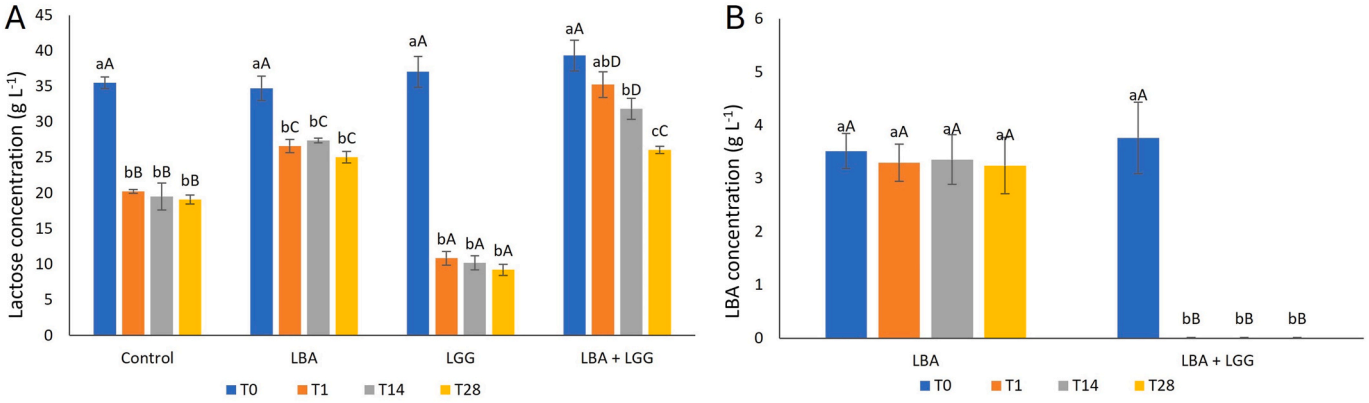

Fig. 4. HPLC analysis of lactose (A) and LBA (B) in fermented milks after the inoculum (T0) and 1, 7, 14, 28 days of storage (T1, T7, T14, T28) at 6 °C. Control: Starter cultures; LBA: Starter cultures + Lactobionic acid; LGG: Starter cultures + *L. rhamnosus* GG; LBA + LGG: Starter cultures + Lactobionic acid + *L. rhamnosus* GG. Within the same timepoint, samples with the same superscript letter must be considered not statistically different ($p > 0.05$). Different lower-case letters indicate significant differences ($p < 0.05$) between different samples with the same shelf life. Different capital letters indicate significant differences ($p < 0.05$) in the same samples during the shelf life.

Table 2
Evaluation of the antagonistic activity of fermented milks against *L. monocytogenes* SCOTT A, *S. aureus* DSM 20231, *E. coli* 555 over refrigerated storage: T1, day 1; T7, day 7; T14, day 14; T28, day 28. Control: Starter cultures; LBA: Starter cultures + Lactobionic acid; LGG: Starter cultures + *L. rhamnosus* GG; LBA + LGG: Starter cultures + Lactobionic acid + *L. rhamnosus* GG.

	L. monocytogenes SCOTT A				*S. aureus* DSM 20231				*E. coli* 555			
	T1	T7	T14	T28	T1	T7	T14	T28	T1	T7	T14	T28
Control	- [a]	–	+ [b]	+	–	–	+	+	–	–	+	+
LBA	–	–	+	+	–	–	+	+	–	–	+	+
LGG	++	++ [c]	++	+++ [d]	++	++	++	+++	++	++	++	++
LBA + LGG	++	++	++	+++	++	++	++	+++	++	++	++	++

The diameter of inhibition, for each strain, was the average of three replicates.
[a] no inhibition.
[b] inhibition 1–3 mm.
[c] inhibition 3–6 mm.
[d] inhibition >6 mm.

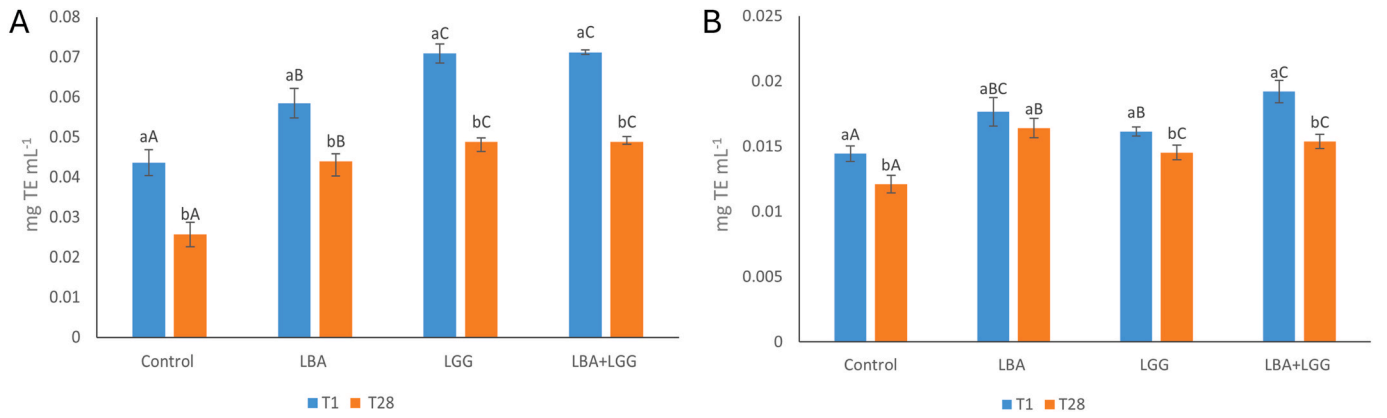

Fig. 5. ABTS (A) and DPPH (B) scavenging ability of the fermented milk samples after 1 and 28 days of storage (T1 and T28). Control: Starter cultures; LBA: Starter cultures + Lactobionic acid; LGG: Starter cultures + *L. rhamnosus* GG; LBA + LGG: Starter cultures + Lactobionic acid + *L. rhamnosus* GG. Different lower case letters indicate significant differences ($p < 0.05$) between the same samples during the shelf life. Different capital letters indicate significant differences ($p < 0.05$) between different samples with the same shelf life.

was metabolized by *L. rhamnosus* GG. Indeed, the LBA at low concentrations may be metabolized by *Lactobacillus* spp. (Sáez-Orviz et al., 2020a), including *L. rhamnosus* strains, as clearly shown by Saarela et al. (2003), underlining the prebiotic potential of LBA. On the other hand, García et al. (2017, 2019) reported that *Lacticaseibacillus casei* CECT 475 did not consume LBA in presence of lactose, but only when the acid sugar was the sole carbon source. In general, studies regarding the metabolism of lactic acid bacteria in presence of both LBA and lactose are limited.

3.4. Antagonistic activity of fermented milks against food spoilage strains

Fermented milks were tested for their antagonistic activity against selected food spoilage strains such as *L. monocytogenes* SCOTT A, *S. aureus* DSM 20231, *E. coli* 555 (Table 2). Fermented milks containing only starter cultures (control) and LBA showed moderate antagonistic activity against the three strains starting at 14 days and this activity remained stable for 28 days of refrigerated storage. Fermented products containing *L. rhamnosus* GG, either alone or in combination with LBA, showed an increase in antagonistic activity already from the first day of storage, which remained constant over the 28-day storage period for *E. coli* 555, while it was enhanced at 28 days for *L. monocytogenes* SCOTT A, and *S. aureus* DSM 20231. Overall, the better performances of fermented milks containing LGG may depend on the metabolites that the probiotic can produce and accumulate in the product, such as organic acids, hydrogen peroxide, etc. (Tang et al., 2023). In this context, it is worth to notice that the antagonistic activity was maintained regardless the carbon source consumed by the bacteria in these samples (LBA or lactose in LBA + LGG and LGG sample, respectively).

3.5. Antioxidant activity of fermented milks

The antioxidant activity of the fermented milk samples was evaluated by ABTS and DPPH assays (Fig. 5A and B). After 1 day of storage (T1), ABTS scavenging activity values were significantly higher in all samples compared to control (Fig. 5A), especially when *L. rhamnosus* GG was present. Although with an overall reduction, the same behavior was observed on samples at 28 days of storage. Regarding the DPPH assay (Fig. 5B), the data analysis reveals trends that are partially consistent with the ABTS results. After one day of storage, DPPH values were significantly higher for all samples compared to the control. However, in this case, samples containing LBA exhibited significantly higher values. When the 28-day shelf life was reached, the greatest reduction was observed in the control, while lower reductions were observed in LGG and LBA + LGG. In contrast, it is interesting to note that the LBA sample retained its antioxidant activity better than the other samples even after 28 days of storage, confirming the antioxidant effect of LBA (Alonso et al., 2011; Cardoso et al., 2019; Coroli et al., 2021; Goderska, 2019b).

Table 3
Volatile organic compounds of milk samples, analyzed in pre-fermentation phase, with or without LBA in order to better assess the initial impact of the addition of LBA. Data expressed in peak area ratio (PAR) and defined as the ratio of the analyte peak area (Area compound) to the internal standard peak area (Area IS), are the mean values of three replicates. Standard deviation observed ranged between 3 and 5 %.

Pre-fermentation phase		
Peak area ratio (PAR)	*Milk*	*Milk + LBA*
Acetone	1.20	1.14
Ethyl Acetate	0.18	0.21
2-Butanone	0.64	0.64
Ethanol	0.40	0.44
2-Pentanone	0.25	0.34
Methyl Isobutyl Ketone	0.32	0.26
Toluene	- [a]	0.06
beta.-Myrcene	0.02	0.04
2-Heptanone	0.27	0.35
1-Butanol, 3-methyl-	0.06	–
D-Limonene	0.24	0.60
Acetoin	0.09	0.15
1-Hexanol	0.10	0.07
2-Nonanone	0.04	0.07
Acetic acid	0.05	0.05
1-Hexanol, 2-ethyl-	0.02	0.02
1,6-Octadien-3-ol, 3,7-dimethyl-	0.01	–
2-Undecanone	–	0.01
Butanoic acid	0.02	0.02
2-Furanmethanol	0.02	0.10
Oxime-, methoxy-phenyl-_	0.05	–
Hexanoic acid	0.03	0.03
Dimethyl sulfone	0.02	0.03
Phenol	0.01	0.01
2(3H)-Furanone, dihydro-5-pentyl-	0.01	0.01
Octanoic acid	0.01	0.02
Nonanoic acid	0.01	0.01
n-Decanoic acid	–	0.01
Octaethylen glycole	0.02	–

[a] Means not detected.

3.6. Volatile organic compounds of fermented milks

The sensory properties of fermented milks, especially in the context of the addition of LBA, are a key aspect influencing consumer choice. As already mentioned in the Introduction, the few studies available on the

Table 4
Volatile organic compounds of fermented milk samples at the beginning (T1) and at the end of storage period (T28) at 6 °C. Data expressed in peak area ratio (PAR), defined as the ratio of the analyte peak area (Area compound) to the internal standard peak area (Area IS), are the mean values of three replicates. Standard deviation observed ranged between 3 and 5 %. Control: Starter cultures; LBA: Starter cultures + Lactobionic acid; LGG: Starter cultures + *L. rhamnosus* GG; LBA + LGG: Starter cultures + Lactobionic acid + *L. rhamnosus* GG.

Peak area ratio (PAR)	T1 - Control	T1 - LBA	T1 - LGG	T1 - LBA + LGG	T28 - Control	T28 - LBA	T28 – LGG	T28 - LBA + LGG
n-Hexane	- [a]	–	–	–	–	0.46	–	–
Acetone	0.31	0.28	–	–	0.19	0.35	–	–
2-Butanone	0.18	0.17	–	–	0.10	0.19	–	–
Ethanol	–	–	0.45	0.48	–	–	0.79	0.63
2-Pentanone	–	–	0.06	0.05	–	–	–	–
Acetic acid ethenyl ester	0.60	0.63	–	–	0.93	1.92	–	–
2,3-Pentanedione	0.10	0.11	–	–	0.13	0.28	–	–
2-Heptanone	0.26	0.23	0.13	0.13	0.13	0.35	–	–
Acetoin	0.31	0.25	–	–	0.19	0.46	–	–
2-Heptanol	–	–	0.03	0.04	–	–	0.13	0.13
2-Propanone, 1-hydroxy-	–	–	–	–	–	0.11	0.00	0.00
2-Buten-1-ol, 3-methyl-	–	–	0.01	0.01	–	–	0.02	0.02
Glycolaldehyde dimer	–	–	–	–	–	0.11	–	–
1-Hexanol	0.04	0.04	0.01	0.01	0.03	0.00	0.01	0.01
Propanoic acid, 2-hydroxy-2-methyl- methyl ester	–	–	–	–	–	0.06	–	–
2-Hydroxy-3-pentanone	0.02	0.02	–	–	0.02	0.03	–	–
2-Nonanone	0.05	0.05	0.02	0.02	0.02	0.08	0.01	0.00
Acetic acid	0.04	0.07	0.23	0.27	0.08	0.55	1.28	1.08
Furfural	–	0.01	–	–	–	0.17	0.01	0.00
2-Nonanol	–	–	0.01	0.01	–	–	0.02	0.02
Ethene, fluoro-	–	–	–	–	–	0.34	0.07	0.04
2-Undecanone	0.02	0.01	0.01	0.01	0.01	0.02	–	–
Butanoic acid	0.05	0.04	0.05	0.06	0.06	0.17	0.12	0.09
Butanoic acid, 4-hydroxy-	0.00	–	–	–	–	0.02	–	–
2-Furanmethanol	0.04	0.08	0.01	0.05	0.02	0.72	0.01	0.04
2-Undecanol	–	–	–	–	–	–	0.01	0.01
1,2-Cyclopentanedione	–	–	–	–	–	0.04	0.00	–
Hexanoic acid	0.07	0.05	0.07	0.09	0.09	0.23	0.16	0.13
Phenylethyl Alcohol	0.01	0.01	–	–	–	–	–	–
Maltol	–	–	–	–	–	0.02	–	–
2-Hydroxymethyl-2-methyl-pyrrolidine-1-carboxaldehyde	–	–	–	–	–	0.07	–	–
Phenyl-.beta.-D-glucoside	–	–	–	–	–	0.02	–	–
Methyl 2-furoate	–	–	–	–	–	0.02	–	–
2,5-Dimethyl-4-hydroxy-3(2H)-furanone	–	–	–	–	–	0.01	–	–
Octanoic acid	0.03	0.03	0.04	0.04	0.03	0.10	0.06	0.05
Dihydroxyacetone	–	–	–	–	–	0.05	–	–
1,4-Butanediol	–	–	–	–	–	0.07	–	–
1,2,6-Hexanetriol	–	–	–	–	–	0.03	–	–
2-Hydroxy-gamma-butyrolactone	–	–	–	–	–	0.04	–	–
Nonanoic acid	–	–	0.01	–	–	–	–	–
4-Hydroxy-2-methylacetophenone	–	–	0.01	–	–	–	–	–
n-Decanoic acid	–	–	0.01	0.01	0.01	0.04	0.01	0.01
Benzoic acid	–	–	–	0.02	0.02	0.18	0.09	0.07
5-Hydroxymethylfurfural	–	–	–	–	–	0.21	–	–
Beta-hydroxy-gamma-butyrolactone	–	–	–	–	–	0.21	–	–

[a] Means not detected.

use of LBA added as a food ingredient focus mainly on the improvement of technological properties and disregard the sensory effects in terms of volatile molecule profile. According to Cardoso et al. (2019) the sensory properties of cheese produced with LBA as an ingredient are considered satisfactory and fully suitable according to a patented process for cheese and other dairy products (U.S. Patent No. 7329424 B2, 2001). According to Kiryu et al. (2009a) and Ribeiro et al. (2016), LBA also has the potential to preserve the aroma, including the ability to mask off-flavors in Caspian Sea yogurt produced with *A. orientalis*. In this context, the limitations in the use of LBA, which may also occur during product analysis by a panel test, can be at least partially overcome through a preliminary instrumental analysis, such as GC-MS-SPME, to determine VOCs. Volatile compounds were identified both in pre-fermented samples to evaluate the potential impact of LBA-enriched whey (Table 3), and in all samples after fermentation and/or during storage (Table 4). Although each fermented product was characterized by a specific aroma profile with different molecules, compounds belonging to alcohols, ketones, and organic acids were detected in all the samples (Tables 3 and 4). Our results show that the volatile molecules changed only slightly in the pre-fermentation phase after the addition of LBA-enriched cheese whey. However, after fermentation, and especially with the progression of refrigerated storage (T28), differences were observed between the samples. To better understand the effects of the variables adopted (LBA, LGG, LBA + LGG) on the volatilome of the fermented products, a principal component analysis (PCA) was performed using the volatilome data. The projection of the samples and the related molecules, analyzed after 1 and 28 days of storage (Fig. 6A and B), were able to explain 88.68 % of the total variance among the samples. As already mentioned, Table 3 shows that, on the first day of storage, control and LBA clustered together while LGG and LBA + LGG created another opposite clustered (Fig. 6A). Instead, at the end of shelf-life (T28), LBA sample significantly differentiated from the other experimental conditions. The observed differences are primarily attributed to the presence of specific aromatic compounds only in this sample, such as maltol, 2-hydroxymethyl-2-methyl-pyrrolidine-1-carboxaldehyde, phenyl-β-D-glucoside, methyl 2-furoate, 2,5-dimethyl-4-hydroxy-3(2H)-furanone, dihydroxyacetone,

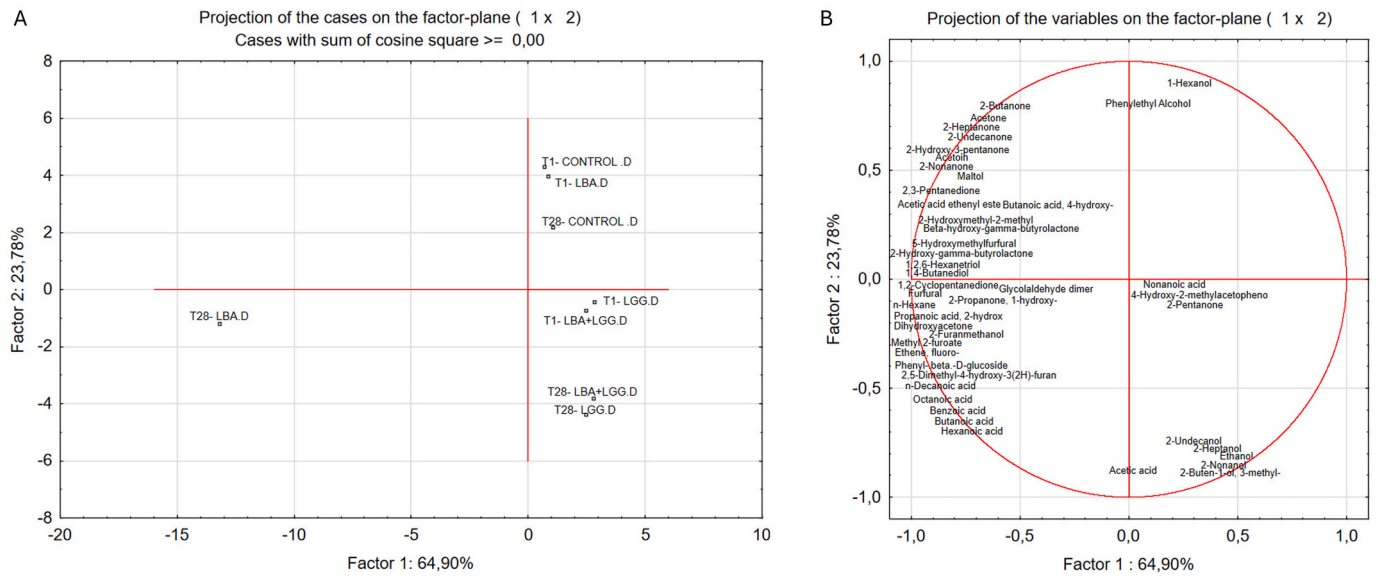

Fig. 6. Plot of cases (A) and variables (B) obtained by PCA elaboration of the total volatile molecules characterizing the fermented milk samples after 1 day and 28 days of storage at 6 °C. Control: Starter cultures; LBA: Starter cultures + Lactobionic acid; LGG: Starter cultures + *L. rhamnosus* GG; LBA + LGG: Starter cultures + Lactobionic acid + *L. rhamnosus* GG.

2-hydroxy-γ-butyrolactone, 5-hydroxymethylfurfural, and β-hydroxy-γ-butyrolactone (Table 4, Fig. 6B.) It is interesting to note that some of these compounds, such as maltol, 2,5-dimethyl-4-hydroxy-3(2H)-furanone, 2-hydroxy-gamma-butyrolactone and 5-hydroxymethylfurfural, originate from the Maillard reaction and are generally associated with sweet, caramel-like notes (Paravisini et al., 2014; Zeng et al., 2023). In particular the last two molecules likely derived from the thermal treatment of LBA cheese whey. These above-mentioned compounds were neither found in the LBA sample during the pre-fermentation phase or at T1, nor in the LBA + LGG sample at the analyzed time points (T1 and T28). These compounds are therefore probably related to the addition of the LBA-enriched cheese whey but mainly generated during storage. The same compounds at T28, however, could be utilized by the LGG in the LBA + LGG sample, as also shown by the data on LBA utilization in previous sections. Furthermore, by referring to the data shown in Table 4 and Fig. 6B, it is evident that the samples containing LGG are primarily characterized by the presence of acetic acid, which increases as refrigerated storage progresses. This compound, produced through the heterofermentative metabolism of *L. rhamnosus* and released into the product, could enhance its antimicrobial effect, as shown in Table 2. However, high levels of acetic acid (which imparts sour, vinegar-like notes) may negatively impact the organoleptic properties and overall acceptability of the fermented milk. Typically, acetic acid is present in commercial yogurts at concentrations ranging from 0.5 to 18.8 mg kg^{-1} (Alonso & Fraga, 2001), which is consistent with the levels observed in our products. Overall, it is important to note that SPME is a technique capable of providing aromatic fingerprints that closely resemble those perceived by consumers. Nevertheless, final sensory perception is not solely determined by the concentration of individual volatile compounds. Instead, it is the result of complex interactions and dynamic equilibria among volatile and non-volatile molecules within the food matrix. Structural and microstructural properties of the product, along with interactions involving non-volatile components such as proteins and polysaccharides, can significantly influence both the release and perception of aroma compounds. Therefore, interpreting sensory quality based only on the concentration of a single volatile compound may be misleading. Indeed, certain molecules—despite being present at very low concentrations—can have a pronounced impact on aroma perception due to their low odor thresholds and high olfactory potency. Indeed, further panel tests with trained panelists or consumers are needed to define the final acceptability of the product.

4. Conclusions

To align with sustainable development goals and the principles of the circular economy, this study evaluated the use of LBA-enriched cheese whey in fermented milk formulations by incorporating it directly, without purification steps, thereby reducing additional costs and valorizing a dairy by-product. The incorporation of LBA-enriched cheese whey into fermented milk formulations promoted the growth of *L. rhamnosus* GG and enhanced the antioxidant activity *in vitro*, suggesting a potential prebiotic role for LBA. Notably, *L. rhamnosus* GG preferentially utilized LBA, even if present at lower concentrations (3 g L^{-1}), over lactose indicating a metabolic shift that may influence the formulation of symbiotic products. This novel finding highlights the importance of selecting probiotic strains that prioritize lactose over LBA. While previous studies have primarily focused on LBA as sole carbon source, this work emphasizes the need to consider its interaction with other substrates such as lactose. However, since LBA was extensively metabolized during fermentation, its availability to the host is questionable, and its prebiotic effect remains to be demonstrated *in vivo*. Further research will explore the impact of these formulations on an *in vitro* model simulatingthe human microbiota, paving the way for subsequent clinical evaluations.

CRediT authorship contribution statement

Margherita D'Alessandro: Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis. **Davide Gottardi:** Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis. **Sara Franceschini:** Writing – review & editing, Investigation, Formal analysis. **Giacomo Braschi:** Investigation. **Lorenzo Siroli:** Investigation. **Lorenzo Nissen:** Writing – review & editing. **Roberta Romano:** Investigation, Formal analysis. **Lorenza Putignani:** Writing – review & editing. **Andrea Gianotti:** Writing – review & editing. **Noura Raddadi:** Writing – review & editing, Validation, Supervision, Resources. **Rosalba Lanciotti:** Writing – review & editing. **Pamela Vernocchi:** Writing – review & editing, Funding acquisition, Conceptualization. **Francesca Patrignani:** Writing – review & editing, Validation, Supervision,

Resources, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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