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Addition of glutamine to milk during fermentation by individual strains of lactic acid bacteria and the effects on pyroglutamic and butyric acid

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ABSTRACT

Lactic acid bacteria fermentation generates pyroglutamic and butyric acid, two metabolites that influence the flavour of foods and could significantly benefit human health. In literature, data on the ability of lactic acid bacteria to produce these molecules is limited. Therefore, in this study, single strains were inoculated in milk to determine the quantity of butyric and pyroglutamic acid produced and the effect of glutamine enrichment substrate was evaluated. In addition to *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, which are used in traditional yoghurt, other strains (including some probiotics) isolated from different sources were studied. *L. bulgaricus* E1 and the probiotic *L. casei* ATCC393 generated the most butyric acid (110 and 108 mg 100 g^{-1} d.m., respectively) in media without glutamine. The highest quantity of pyroglutamic acid was produced by *Lactobacillus delbruekii* subsp. *bulgaricus* MY (700 mg 100 g^{-1} d.m.) and the probiotic *Lacticaseibacillus casei* Shirota (1786 mg 100 g^{-1} d.m.), respectively. Most of the inoculated strains produced a greater quantity of pyroglutamic acid in the substrate with glutamine. These results are an interesting starting point for researchers to utilize selected strains of lactic acid bacteria to develop new dairy products with functional properties due to the presence of pyroglutamic and butyric acid.

1. Introduction

Fermented milk is produced by the coagulation of milk, without eliminating serum, through utilizing starter cultures that generally remain alive and vital until consumption (Macori and Cotter, 2018). Microorganisms especially used in fermented dairy products are lactic acid bacteria (LAB).

Based on the microbial composition, fermented milks can be divided into the following categories: thermophilic sour milks, mesophilic sour milks and alcoholic-acid milks (Cruz et al., 2021). In thermophilic sour milks, fermentation occurs at a temperature of 37–45 °C, and lactic acid is the main fermentation product. Yogurt belongs to this group and is produced using the starter pair *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* (Shiby and Mishra, 2013). On the other hand, mesophilic sour milks are obtained from fermentations carried out at temperatures below 30 °C by homo and heterofermentative microorganisms of the genera *Lactococcus*, *Lactobacillus* and *Leuconostoc* (Tamime, 2002). Acidic-alcoholic milks include kefir, kumys and gioddu; these milks are characterized by the following fermentation products at 15–25 °C: lactic acid, diacetyl, ethyl alcohol and CO_2 (Chandan et al., 2008). Some studies have reported the health benefits of fermented foods, which are typically associated with the presence of microorganisms that can produce several beneficial byproducts/metabolites (Marco et al., 2017; Macori and Cotter, 2018). LAB and their byproducts have been classified with generally recognized as safe status, making them suitable for a wide range of applications (Klaenhammer et al., 2005). LAB possesses numerous metabolic and protechnological properties, including acidifying, proteolytic, and lipolytic activities, as well as antioxidant and flavor-enhancing capacities. In particular, incorporating lactic acid bacteria into milk during dairy-products production can boost the generation of free amino acids, peptides, and aromatic molecules like diacetyl and acetoin (Reale et al., 2016). LAB-driven fermentations often generate potentially bioactive compounds, such as pyroglutamic and butyric acids, and exhibit a wide range of beneficial health effects (Mathur et al., 2020). Many studies highlight the known benefits associated with LAB. LAB are associated

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with cholesterol-lowering and anticancer properties (Kapila et al., 2007). Fermented dairy product consumption may reduce the probability of developing type 2 diabetes mellitus and cardiovascular disease (Chen et al., 2014; Tapsell, 2015). These products are also associated with an improvement in cognitive functions (Tillisch et al., 2013) and are well tolerated by lactose-intolerant individuals because most lactose in the products is fermented by LAB during production (Savaiano, 2014). LAB exhibit an antimicrobial capacity due to the production of some metabolites, such as bacteriocins, which can inhibit the growth of spoilage and pathogenic bacteria in food (Khalid, 2011). Among LAB, there are some probiotic strains that can provide many health benefits; for example, the strains can improve the intestinal mucosa barrier, exhibit antagonistic effects against pathogenic microorganisms, stimulate the immune system, exhibit antitumour and antimutagenic activity and reduce symptoms related to inflammatory bowel diseases (IBDs) (Stanton et al., 2005; Gobbetti et al., 2010; Saez-Lara et al., 2015).

Pyroglutamic and butyric acid are two metabolites produced by lactic acid bacteria fermentation (Aiello et al. 2023a).

Pyroglutamic acid, also known as 5-oxoproline, 2-pyrrolidone 5-carboxylic acid, is the cyclic lactam of glutamic acid or glutamine (Kumar and Bachhawat, 2012). Pyroglutamic acid synthesis can be associated with several metabolic pathways. LAB can produce pyroglutamic acid using enzymes with cyclase activity, including *glutamine cyclase* found in lactic acid bacteria such as *L. helveticus*, *L. delbrueckii* subsp. *bulgaricus*, *St. thermophilus* and *L. delbrueckii* subsp. *lactis* (Mucchetti et al., 2002; Schilling et al., 2004). Pyroglutamic acid can be released by the enzymatic lysis of specific proteins or peptides mediated by aminopeptidases, such as *PYRase* or *pyroglutamyl peptidase* (Mucchetti et al., 2002; Chelius et al., 2006; Lou et al., 2006).

Lactic acid bacteria are a source of esterases and/or lipases; these enzymes can act on the lipid component and form short-chain fatty acids (SCFAs), which mainly include acetate, propionate and butyrate (McSweeney and Sousa, 2000; Holland et al., 2005). According to some authors (Beshkova et al., 1998; Chen et al., 2017), free SCFA production starts from peptides or amino acids. In particular, the synthesis of butyrate could be associated with γ-amino butyric acid (GABA), which originates from glutamate produced by the deamidation of glutamine via glutaminase (Tapiero et al., 2002; Sarasa et al., 2020). However, the butyrogenic capacity of lactic acid bacteria belonging to *Lactiplantibacillus plantarum* may be associated with a metabolic pathway that involves lipase-induced hydrolysis of triglycerides (Aiello et al., 2023b).

L-glutamine is a nonessential amino acid that can be synthesized by the human body (Watford et al., 2000) and does not require supplementation with the diet. Some studies have demonstrated its protective and supportive role, especially in athletes (Piattoly, 2005; Katmawanti et al., 2023). Data on the supplementation of foods with glutamine remains limited. Some authors, such as Gomaa et al. (2018) and Aleman et al. (2023), have evaluated the effect of adding glutamine to dairy products, in particular yoghurt and fermented milk. They found that glutamine fortified yoghurt resulted in chemical, physical and sensory characteristics close or similar to the traditional yoghurt. In addition, some studies highlighted the beneficial effects of glutamine on human body. Castell (2002) reported that in cases of severe stress, muscle trauma and prolonged exercise the concentration of glutamine in the blood decreased significantly. Ceja et al. (2023) showed the positive effect of glutamine on the immune system and health of the gastrointestinal tract. For this reason, it is interesting to consider functional dairy products fortified with glutamine. At the same time, investigating the correlation between glutamine enrichment and lactic acid bacteria fermentation is interesting, aiming to evaluate its impact on the production of other noteworthy molecules, such as pyroglutamic and butyric acid.

Pyroglutamic and butyric acid exhibit significant beneficial effects on human health. Pyroglutamic acid can stop tumour cell growth (Kimura, 2005), prevent the onset of type-2 diabetes (Yoshinari and Igarashi, 2011), exert positive effects on the retina and optic nerve (Oono et al., 2009) and inhibit microbial growth (Yang et al., 1997). Butyric acid is the primary energy source used by epithelial cells in the colon (Donohoe et al., 2011); can prevent the onset of IBD, allergies and autoimmune diseases (Furusawa et al., 2013); promotes colon cancer cell apoptosis (Smith and Workman, 2009) and may exhibit an anti-inflammatory effect (Zhang et al., 2009).

To the best of our knowledge, current data on the ability of LAB to produce these molecules is limited in scientific literature. Some authors (Mucchetti et al., 2000; 2002) have evaluated the presence of pyroglutamic acid in ripened cheeses and its production by thermophilic LAB such as *Lactobacillus helveticus*, *Lactobacillus delbrueckii* and *Streptococcus thermophilus*. More recent works were conducted on the production of pyroglutamic and butyric acid in dairy products. Aiello et al. (2022) evaluated the concentration of pyroglutamic acid in commercially available fermented dairy products and its formation kinetics during the fermentation process; Aiello et al. (2023a) evaluated the influence of probiotics and/or prebiotics on the content of butyric and pyroglutamic acid in yoghurt during the storage period.

Therefore, considering the multiple beneficial effects of pyroglutamic and butyric acid, this study aims to investigate their production in milk by different single strains of lactic acid bacteria belonging to genera *Streptococcus*, *Lactobacillus*, *Lacticaseibacillus* and *Lactiplantibacillus* (including some probiotic and non-probiotic varieties) and to evaluate the effect of glutamine enrichment as a precursor of these two molecules.

2. Materials and methods

2.1. Materials

Fermented milk was produced in triplicate using 10 mL pasteurized fresh milk (3.4% protein, 3.6% fat and 4.8% lactose, as reported on the label) and inoculated with single strains of lactic acid bacteria at a rate of 2% (w/w). The study was conducted on 22 strains of LAB belonging to genera *Streptococcus*, *Lactobacillus*, *Lacticaseibacillus* and *Lactiplantibacillus* as shown in the supplementary Table 1. The frozen strains were individually revitalized in de Man, Rogosa and Sharpe broth (MRS) before inoculation.

All samples (Table 1) were prepared in duplicate by inoculating microorganisms in milk and glutamine-fortified milk. The latter was prepared by adding a known amount of standard (L-glutamine 99%, Sigma—Aldrich, St. Louis, MO, US) to the pasteurized fresh milk before the inoculation, up to a final concentration of 7 mg mL^{-1}. This concentration was chosen based on the methodology outlined by Mucchetti et al. (2002), who utilized 7 mg mL^{-1} of glutamine to assess the cyclase activity of lactic acid bacteria in their study. Two control samples of milk and glutamine-fortified milk without inoculation were used as control (Control and Control+G, respectively). The incubation was carried out at 37 °C in a Panasonic MIR-154-PE incubator (Osaka, Japan) for 48 h.

2.2. Dry matter determination

The pasteurized fresh milk used as substrate was placed in an air oven (Thermo Electron Corporation, Waltham, Massachusetts, USA) to calculate dry matter. For this purpose, 2 g of each sample, in triplicate, was weighed in porcelain dishes and subsequently dried at 102 °C for 2 hours (AOAC, 2005). The results were expressed as percentage weight/weight.

2.3. pH determination

The pH value, which represents the real acidity of the fermented milk as well as the hydrogen ion concentration, was determined in all samples by a pH meter (Medidor pH BASIC 20 Crison Instruments, Barcelona, Spain).

Table 1
Milk (pasteurized fresh milk) fermented with different microbial strains.

Sample	Substrate	Microbial composition
Control	Pasteurized fresh milk	-
Control+G	Pasteurized fresh milk + glutamine	-
T(85)	Pasteurized fresh milk	*Streptococcus thermophilus 85*
T(85)+G	Pasteurized fresh milk + glutamine	*Streptococcus thermophilus 85*
T(50)	Pasteurized fresh milk	*Streptococcus thermophilus 50*
T(50)+G	Pasteurized fresh milk + glutamine	*Streptococcus thermophilus 50*
M(96)	Pasteurized fresh milk	*Streptococcus macedonicus 96*
M(96)+G	Pasteurized fresh milk + glutamine	*Streptococcus macedonicus 96*
M(97)	Pasteurized fresh milk	*Streptococcus macedonicus 97*
M(97)+G	Pasteurized fresh milk + glutamine	*Streptococcus macedonicus 97*
B(MY)	Pasteurized fresh milk	*Lactobacillus delbruekii subsp bulgaricus MY*
B(MY)+G	Pasteurized fresh milk + glutamine	*Lactobacillus delbruekii subsp bulgaricus MY*
B(CCB)	Pasteurized fresh milk	*Lactobacillus delbruekii subsp bulgaricus CCB*
B(CCB)+G	Pasteurized fresh milk + glutamine	*Lactobacillus delbruekii subsp bulgaricus CCB*
B(CPV)	Pasteurized fresh milk	*Lactobacillus delbruekii subsp bulgaricus CPV*
B(CPV)+G	Pasteurized fresh milk + glutamine	*Lactobacillus delbruekii subsp bulgaricus CPV*
B(E1)	Pasteurized fresh milk	*Lactobacillus delbruekii subsp bulgaricus E1*
B(E1)+G	Pasteurized fresh milk + glutamine	*Lactobacillus delbruekii subsp bulgaricus E1*
B(AZP1)	Pasteurized fresh milk	*Lactobacillus delbruekii subsp bulgaricus AZP1*
B(AZP1)+G	Pasteurized fresh milk + glutamine	*Lactobacillus delbruekii subsp bulgaricus AZP1*
*H(52)	Pasteurized fresh milk	*Lactobacillus helveticus Rossell-52*
*H(52)+G	Pasteurized fresh milk + glutamine	*Lactobacillus helveticus Rossell-52*
*A(24)	Pasteurized fresh milk	*Lactobacillus acidophilus DSM 24735*
*A(24)+G	Pasteurized fresh milk + glutamine	*Lactobacillus acidophilus DSM 24735*
*Pl(299)	Pasteurized fresh milk	*Lactiplantibacillus plantarum 299 v*
*Pl(299)+G	Pasteurized fresh milk + glutamine	*Lactiplantibacillus plantarum 299 v*
*C(393)	Pasteurized fresh milk	*Lacticaseibacillus casei ATCC393*
*C(393)+G	Pasteurized fresh milk + glutamine	*Lacticaseibacillus casei ATCC393*
*C(S)	Pasteurized fresh milk	*Lacticaseibacillus casei SHIROTA*
*C(S)+G	Pasteurized fresh milk + glutamine	*Lacticaseibacillus casei SHIROTA*
*R(11)	Pasteurized fresh milk	*Lacticaseibacillus rhamnosus Rossell-11*
*R(11)+G	Pasteurized fresh milk + glutamine	*Lacticaseibacillus rhamnosus Rossell-11*
R(A1G)	Pasteurized fresh milk	*Lacticaseibacillus rhamnosus A1G*
R(A1G)+G	Pasteurized fresh milk + glutamine	*Lacticaseibacillus rhamnosus A1G*
R(A3)	Pasteurized fresh milk	*Lacticaseibacillus rhamnosus A3*
R(A3)+G	Pasteurized fresh milk + glutamine	*Lacticaseibacillus rhamnosus A3*
Pe(13)	Pasteurized fresh milk	*Lactiplantibacillus pentosus 13*
Pe(13)+G	Pasteurized fresh milk + glutamine	*Lactiplantibacillus pentosus 13*
Pe(14)	Pasteurized fresh milk	*Lactiplantibacillus pentosus 14*
Pe(14)+G	Pasteurized fresh milk + glutamine	*Lactiplantibacillus pentosus 14*
Pe(24)	Pasteurized fresh milk	*Lactiplantibacillus pentosus 24*
Pe(24)+G	Pasteurized fresh milk + glutamine	*Lactiplantibacillus pentosus 24*
Pe(50)	Pasteurized fresh milk	*Lactiplantibacillus pentosus 50*
Pe(50)+G	Pasteurized fresh milk + glutamine	*Lactiplantibacillus pentosus 50*
Pe(54)	Pasteurized fresh milk	*Lactiplantibacillus pentosus 54*
Pe(54)+G	Pasteurized fresh milk + glutamine	*Lactiplantibacillus pentosus 54*

*probiotic strain.

2.4. Lactic and pyroglutamic acid determination

2.4.1. Organic acid extraction

Lactic and pyroglutamic acid extraction from all samples was carried out following the method described by Bevilacqua and Califano (1989) with some modifications to prepare the sample for reversed-phase high-performance liquid chromatography (RP-HPLC) analysis. Then, 25 mL 0.5% (w/w) $(NH_4)_2HPO_4$ in bidistilled water (Sigma Aldrich) was added to 3.5 g of each sample in a 50 mL centrifuge tube and stirred on a magnetic plate for 1 h. The sample was subsequently centrifuged in a multispeed centrifuge (PK 131, ALC International Srl, Milano, Italy) at 7000 rpm for 10 minutes. The supernatant was subjected to double filtration, first with filter paper and then through a 0.45-mm PES hydrophilic membrane filter.

2.4.2. HPLC determination of lactic and pyroglutamic acid content

The quantification of lactic and pyroglutamic acid, for all samples, was carried out by HPLC, according to the method reported by Aiello et al. (2022). Twenty microlitres of each extract was injected into an HPLC (Agilent 1200 series) equipped with a quaternary pump, G4225A degasser, DAD G1315B and FLD G1221A detectors, and a C18 reversed-phase column (Eclipse XDB, 5 μm, 4.6 mm×150 mm). Analysis was carried out isocratically using a mixture of water:methanol:trifluoracetic acid (97.7:2.2:0.1) (pH 1.73) as the mobile phase, with a flow rate of 0.75 mL min^{-1} and 20 min of total run time. The detector wavelength was set at 210 nm. Calibration curves were constructed using different concentrations of lactic and pyroglutamic acid standards (50, 100, 200, 500, 1000 ppm) in $(NH_4)_2HPO_4$ buffer at pH 2.24. The extraction efficiency, which was evaluated through the determination of the recovery of lactic and pyroglutamic acid, was approximately 98.4%. The results were expressed as g kg^{-1} and mg 100 g^{-1} of dry matter for lactic and pyroglutamic acid, respectively.

2.5. Determination of free butyric acid and other volatile fatty acids

2.5.1. SPME

Free short-chain fatty acid (SCFA) extraction was carried out in all samples using the solid phase microextraction (SPME) technique, according to the method described by Lee et al. (2003) with some modifications. Briefly, 2 g of fermented milk was transferred to a 4 mL glass vial, and 1 g of sodium chloride and 15 μl of 2-methyl-3-heptanone (10 mg L^{-1}) were added as internal standards. Once the vial was sealed, the samples were homogenized and heated on a heating magnetic stirrer at 50 °C for 10 minutes. Subsequently, the solid-phase microextraction (SPME) device equipped with a 50–30 μm thickness divinyl-benzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibre coated with a 2 cm length stationary phase was hermetically inserted into the vial containing the sample and left for 1 hour at 50 °C.

2.5.2. Determination of SCFA through GC/MS

The analysis and quantification of volatile SCFAs were performed by gas chromatography coupled with mass spectrometry (GC/MS), according to a method described by Manzo et al. (2019), with some modifications. The SPME fibre was introduced into the GC injector, and thermal desorption of the analytes was performed at 250 °C for 10 minutes in splitless mode. The system used was a 6890 N GC equipped with a 5973-mass detector. Free volatile fatty acids were separated on a 30 m x 0.250 mm capillary column coated with a 0.25 μm film of 95% phenyl and 5% dimethylpolysiloxane. The column oven temperature was programmed at 6 °C/min from an initial temperature of 40 °C (held for 2 min) to 160 °C and then at 10 °C/min to 210 °C, which was held for 10 min. The injection and ion source temperatures were 250 °C and 230 °C, respectively. The carrier gas, helium (He), was used at a 1 mL/min

flow rate. The ionizing electron energy was 70 eV, and the mass range scanned was 40–450 amu in full-scan acquisition mode. Analytes were identified using NIST Atomic Spectra Database version 1.6 and verified by retention rates. The same procedure was carried out on standard aqueous solutions of butyric, caproic and caprylic acid, with different concentrations (10, 50, 100, 250 and 500 mg L^{-1}), to construct calibration curves. The results were expressed as mg 100 g^{-1} of dry matter.

2.6. Statistical analysis

All analyses and determinations were performed in triplicate, and the reported results are the average values obtained ± standard deviation. Data were subjected to one-way analysis of variance (ANOVA) and Tukey's multiple range test ($p \leq 0.05$) using XLSTAT software (Addinsoft, New York, USA).

3. Results and discussion

3.1. Assessment of pH and lactic acid content in fermented milk

Dry matter was 12.12% in pasteurized milk and 10.91% in pasteurized milk with glutamine; these values are within the range of 7–14% reported in the literature (Debon et al., 2010; Akin and Ozcan, 2017). The decrease of dry matter values when glutamine is added was also observed by Gomaa et al. (2018) who reported a higher moisture value in yoghurt with the addition of glutamine up to 3%.

The analysed samples showed an average pH value (Tables 2**a**-2**d**) ranging from 3.64 to 4.53 for milk inoculated with *L. bulgaricus* MY coded B(MY), and milk inoculated with *St. thermophilus* 50 with added glutamine coded T(50)+G, respectively, corresponding with the range of 4.00–4.60 reported in the literature (Mucchetti et al., 2002; Østlie et al., 2003; Aryana and McGrew, 2007). Normally, pasteurized milk has pH values between 6.5 and 6.8 (Fauziah et al., 2020; Tadjine et al. 2021). The samples of the uninoculated milk control, without and with the addition of glutamine, showed pH values of 4.16 and 4.43, respectively, as we expected after incubation at 37°C for 48 h.

Lactic acid is the main fermentation product of lactose in milk and dairy products. In addition, lactic acid influences the flavour (leading to acidic and refreshing tastes) and helps extend the shelf life (preventing the development of putrefactive bacteria), digestibility of casein, absorption of mineral salts and pH and regularity of bowel movements (Fernandez-Garcia and McGregor, 1994). To produce good quality fermented milk, the concentration of lactic acid should be approximately 8 g/L of the fresh product (Casarotti et al., 2014); the range can vary according to the microbial cultures and the fermentation conditions, in particular the incubation time and temperature. Table 2 **a-d** show the lactic acid content of the samples analysed, which varies from a minimum of 29.78 g kg^{-1} in *PL(299)+G to a maximum of 104.99 g kg^{-1} in *H(52)+G and corresponds with reports by various sources in the literature (Østlie et al., 2003). The control samples (without and with glutamine) compared with all groups of microorganisms, showed the lowest concentration of lactic acid, with values of 17.80 and 21.16 g kg^{-1}, respectively, corresponding with reports by Aiello et al. (2019). Among the micro-organisms of the genus *Streptococcus*, M(97) sample produced the greatest amount of lactic acid (Table 2**a**). As reported in the literature, these micro-organisms, which are typical of dairy products, are easily adapted to carry out homo-lactic fermentations (Pacini et al., 2006; De Vuyst and Tsakalidou, 2008). The strain of *L. helveticus* Rossel-52 in *Lactobacillus* group (Table 2**b**) and *Lcb. rhamnosus* Rossell-11 of the *Lacticaseibacillus* group (Table 2**c**), showed the highest lactic acid values (104.99 and 98.33 g kg^{-1} d.m.) in the *H(52)+G and *R(11)+G. The obtained results correspond with those reported by Røssland et al., (2005), who studied the production of antimicrobial compounds (acetic and lactic acid) during LAB fermentation in milk and observed that *Lcb. rhamnosus* produced the greatest amount of lactic acid (10585 mg kg^{-1} wet weight) after 48 hours of incubation at 37 °C. Lactic

Table 2
Values of pH and lactic acid (LA) content in fermented milk without and with glutamine (+G) and inoculated with micro-organisms of *Streptococcus* (a), *Lactobacillus* (b), *Lacticaseibacillus*(c) and *Lactiplantibacillus* genus (d). Control sample was the uninoculated milk.

a) *Streptococcus* fermented milk		
Sample	**pH**	**LA (g kg^{-1} d.m.)**
control	4.16 ± 0.04^{d}	17.80 ± 0.06^{d}
control+G	4.43 ± 0.04^{b}	21.16 ± 0.33^{d}
T(85)	4.48 ± 0.01^{ab}	31.16 ± 2.61^{c}
T(85)+G	4.49 ± 0.01^{ab}	33.32 ± 0.81^{bc}
T(50)	4.51 ± 0.01^{ab}	34.17 ± 1.81^{bc}
T(50)+G	4.53 ± 0.02^{a}	34.88 ± 1.20^{bc}
M(96)	4.46 ± 0.01^{ab}	31.60 ± 1.22^{bc}
M(96)+G	4.50 ± 0.01^{ab}	32.69 ± 1.21^{bc}
M(97)	4.29 ± 0.01^{c}	36.97 ± 0.98^{ab}
M(97)+G	4.04 ± 0.01^{e}	42.63 ± 2.22^{a}
b) *Lactobacillus* fermented milk		
Sample	**pH**	**LA (g kg^{-1} d.m.)**
control	4.16 ± 0.04^{bc}	17.80 ± 0.06 ^{h}
control+G	4.43 ± 0.04^{a}	21.16 ± 0.33 ^{h}
B(MY)	3.64 ± 0.02^{j}	50.06 ± 0.66 ^{g}
B(MY)+G	3.77 ± 0.01^{ghi}	57.93 ± 1.30^{fg}
B(CCB)	4.07 ± 0.01^{de}	70.84 ± 1.59^{cde}
B(CCB)+G	4.06 ± 0.01^{de}	79.28 ± 2.45^{bcd}
B(CPV)	4.00 ± 0.01^{e}	69.45 ± 6.96^{def}
B(CPV)+G	4.11 ± 0.01 ^{cd}	80.85 ± 0.28^{bcd}
B(E1)	4.22 ± 0.03^{b}	65.78 ± 2.04^{ef}
B(E1)+G	3.99 ± 0.01^{e}	75.08 ± 0.99^{bcde}
B(AZP1)	3.88 ± 0.01 ^{f}	78.73 ± 0.57^{bcd}
B(AZP1)+G	3.79 ± 0.01 ^{g}	85.50 ± 1.52^{b}
***H(52)**	3.70 ± 0.01^{ij}	82.59 ± 9.05^{bc}
***H(52)+G**	3.74 ± 0.02^{ghi}	104.99 ± 2.03^{a}
***A(24)**	3.71 ± 0.01^{hij}	52.24 ± 0.61 ^{g}
***A(24)+G**	3.79 ± 0.01^{gh}	58.19 ± 1.73^{fg}
c) *Lacticaseibacillus* fermented milk		
Sample	**pH**	**LA (g kg^{-1} d.m.)**
control	4.16 ± 0.04^{b}	17.80 ± 0.06 ^{g}
control+G	4.43 ± 0.04^{a}	21.16 ± 0.33 ^{g}
***C(393)**	3.79 ± 0.01^{d}	64.07 ± 0.56^{de}
***C(393)+G**	3.83 ± 0.01^{d}	58.34 ± 2.43^{ef}
***C(S)**	3.70 ± 0.03^{ef}	74.44 ± 0.19^{c}
***C(S)+G**	3.68 ± 0.02 ^{f}	69.93 ± 0.92 ^{cd}
***R(11)**	3.67 ± 0.01 ^{f}	89.48 ± 1.96^{b}
***R(11)+G**	3.67 ± 0.01^{de}	98.33 ± 1.59^{a}
R(A1G)	4.01 ± 0.01^{c}	55.29 ± 0.34 ^{f}
R(A1G)+G	4.00 ± 0.01^{c}	68.47 ± 0.07^{d}
R(A3)	4.01 ± 0.01^{c}	54.13 ± 0.97 ^{f}
R(A3)+G	4.06 ± 0.01^{c}	66.42 ± 3.51^{d}
d) *Lactiplantibacillus* fermented milk		
Sample	**pH**	**LA (g kg^{-1} d.m.)**
control	4.16 ± 0.04^{c}	17.80 ± 0.06 ^{h}
control+G	4.43 ± 0.04^{a}	21.16 ± 0.33 ^{h}
***PL(299)**	3.87 ± 0.02 ^{g}	50.70 ± 2.13 ^{cd}
***PL(299)+G**	3.98 ± 0.01 ^{f}	29.78 ± 1.38 ^{g}
PE(13)	3.89 ± 0.01 ^{g}	48.09 ± 1.93^{de}
PE(13)+G	4.31 ± 0.02^{b}	49.39 ± 1.23 ^{cd}
PE(14)	4.01 ± 0.01^{def}	53.90 ± 3.58^{bcd}
PE(14)+G	4.20 ± 0.01^{c}	39.14 ± 1.33 ^{f}
PE(24)	4.06 ± 0.01^{de}	42.49 ± 0.81^{ef}
PE(24)+G	4.16 ± 0.01^{c}	55.45 ± 2.53^{bc}
PE(50)	3.99 ± 0.01^{ef}	54.12 ± 0.52^{bcd}
PE(50)+G	4.08 ± 0.02^{d}	67.69 ± 1.28^{a}
PE(54)	4.01 ± 0.01^{def}	53.74 ± 0.43^{bcd}
PE(54)+G	4.19 ± 0.01^{c}	59.01 ± 0.24^{b}

[a-e]different superscript letters in the same column indicate statistically significant differences ($p < 0.05$).*probiotic strain, a-hdifferent superscript letters in the same column indicate statistically significant differences ($p < 0.05$).
*probiotic strain, a-jdifferent superscript letters in the same column indicate statistically significant differences ($p < 0.05$).
*probiotic strain, a-gdifferent superscript letters in the same column indicate statistically significant differences ($p < 0.05$).
*probiotic strain, [a-h]different superscript letters in the same column indicate statistically significant differences ($p < 0.05$).

acid values of the genus *Lactiplantibacillus* are shown in Table 2**d**; in this group, the sample Pe(50)+G contains the highest value of lactic acid (67.69 g kg^{-1}). Interestingly, probiotic microorganisms, such as *L. helveticus*, *Lcb. rhamnosus*, *Lcb. casei* and *L. acidophilus*, efficiently formed lactic acid from lactose in milk, with concentrations higher than the average of the samples analysed, consistent with data reported in the literature (Østlie et al., 2003; Casarotti et al., 2014; Pinto et al., 2020).

3.2. Pyroglutamic acid content in fermented milk

Fig. 1 (**a-d**) show the pyroglutamic acid (pGlu) content in milk samples inoculated with LAB strains belonging to the different genera, without and with glutamine enrichment. The control and control+G samples show the lowest values of pyroglutamic acid (186.55 and 206.22 mg 100 g^{-1} d.m., respectively) in both *Streptococcus* (Fig. 1**a**) and *Lactiplantibacillus* groups (Fig. 1**d**). The natural presence of pyroglutamic acid in milk probably results from the presence of bacterial enzymes released by bacterial lysis due to heat treatment (Corradini, 1995), which can use glutamine as a substrate for cyclization to pyroglutamic acid. Within the microorganisms of the *Streptococcus* genus (Fig. 1**a**), only samples with glutamine exhibited higher pyroglutamic acid concentration compared to the control. Among the *Lactobacillus* group (Fig. 1**b**), the microorganism that produced the greatest amount of pyroglutamic acid, also in absence of glutamine, was the MY strain of *L. delbruekii subsp. bulgaricus* coded B(MY) with a final concentration of 699.56 mg 100 g^{-1} d.m. after 48 hours at 37 °C. This result corresponds with the data obtained by Aiello et al. (2022), who studied the pGlu content in samples of traditional yogurt created on a laboratory scale using the starter *L. delbruekii subsp. bulgaricus*, reported values of approximately 188.47–403.56 mg 100 g^{-1} (d.m.). In glutamine-enriched samples, most of the inoculated strains, such as T (85)+G, T(50)+G, M(96)+G, M(97)+G, B(MY)+G, B(CPV)+G, *C (393)+G, R(A3)+G and *PL(299)+G produced more pyroglutamic acid than that of the same unenriched substrate. Probiotic strains *Lcb. casei* Shirota (*C(S)+G) and *Lcb. casei* ATCC393 (*C(393)) produced the highest amount (1785.65 and 1473.14 mg 100 g^{-1} d.m., respectively) in *Lacticaseibacillus* group reported in Fig. 1**c**. These results confirm the hypothesis that some LAB strains potentially hold enzymes, such as glutamine cyclase, which use glutamine as a substrate for cyclization to pyroglutamic acid, as reported by Mucchetti et al. (2000) and Liu et al. (2011). Notably, strains *H(52), *A(24) and *R(11) generated almost no pGlu in fermented milk, even with the addition of glutamine (Fig. 1**b**; Fig. 1**c**); these results can be attributed to the strong proteolytic capacity of the microorganisms used and their low capacity for pyroglutamic acid synthesis when used individually. In particular, the probiotic microorganism *L. acidophilus* DSM 24735 is associated with good proteolytic activity due to the presence of many intracellular exopeptidases, such as X-prolyl dipeptydyl and α-aminoacyl-peptide hydrolase, which are responsible for the main N-terminal proteolytic activities (Machuga and Ives, 1984; Brzozowski et al., 2009). Fig. 1**d** shows pyroglutamic acid values of the *Lactiplantibacillus* group compared to non-inoculated milk. The probiotic *Lpb. plantarum* (*PL299) produces a high amount of pyroglutamic acid especially in the presence of glutamine (840.88 mg 100 g^{-1}), as it is a good producer of primary and secondary metabolites including pGlu (Sangmanee and Hongpattarakere, 2014). Among the five strains of *Lpb. pentosus*, namely PE(13), PE(14), PE(24), PE(50), and PE(54), no statistically significant differences were observed in both conditions—without and with the addition of glutamine.

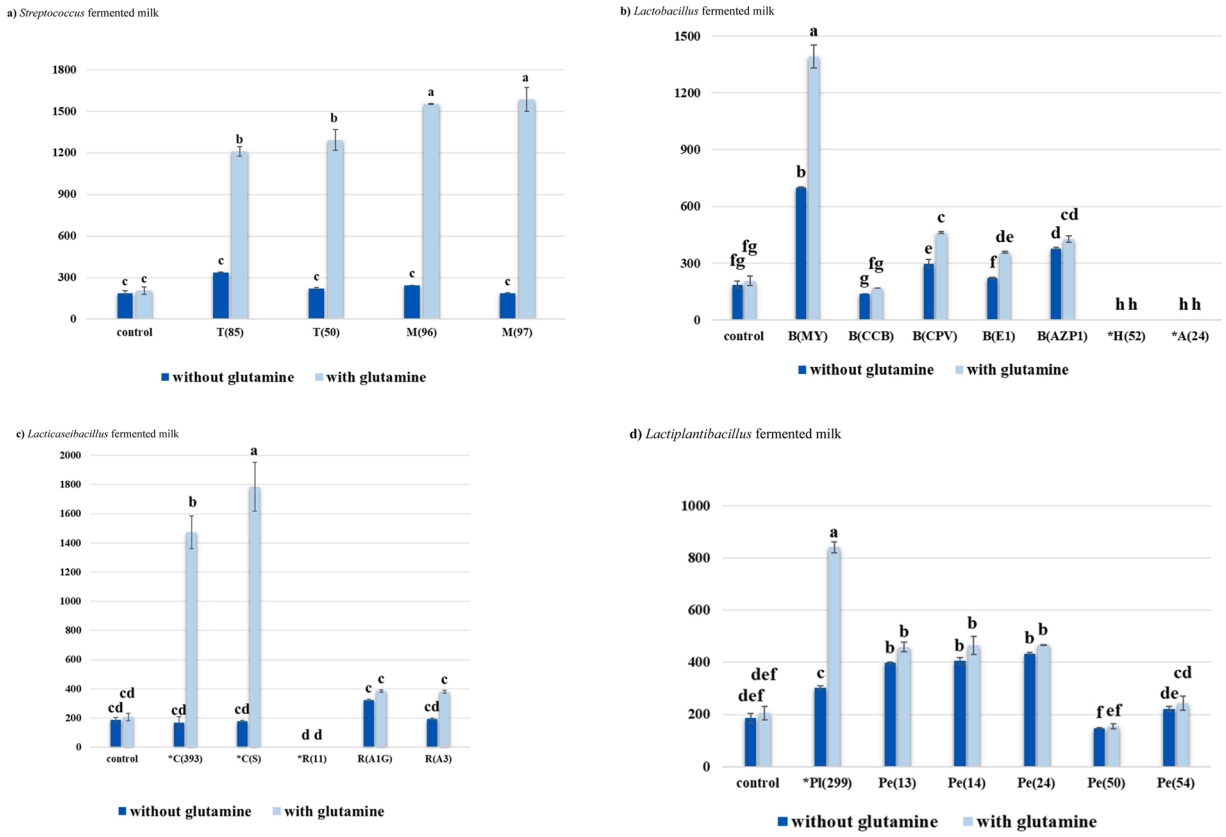

Fig. 1. Pyroglutamic acid content (mg 100 g^{-1}d.m.) in fermented milk without and with glutamine (+G) and inoculated with micro-organisms of *Streptococcus* (a), *Lactobacillus* (b), *Lacticaseibacillus*(c) and *Lactiplantibacillus* genus (d). Control sample was the uninoculated milk. **a)** *Streptococcus* fermented milk. $^{a\text{-}c}$different letters on the bars indicate statistically significant differences ($p < 0.05$). **b)** *Lactobacillus* fermented milk. *probiotic strain, $^{a\text{-}h}$different letters on the bars indicate statistically significant differences ($p < 0.05$). **c)** *Lacticaseibacillus* fermented milk. *probiotic strain, $^{a\text{-}d}$ different letters on the bars indicate statistically significant differences ($p < 0.05$). **d)** *Lactiplantibacillus* fermented milk. *probiotic strain, $^{a\text{-}f}$ different letters on the bars indicate statistically significant differences ($p < 0.05$).

To date, there are no official recommendations on daily doses of pyroglutamic acid to receive beneficial health effects. Some studies in the literature have shown that the amount of these molecules and their beneficial effects on health are correlated. According to Grioli et al. (1990), a dose of 3 g/day pyrglutamic acid administered in the form of arginine salt has been shown to exhibit positive effects on memory loss associated with advancing age. More recently, Pfeiffer and König, (2009) observed that the recommended dose of pyrglutamic acid to achieve improvements in memory and learning is 400–1000 mg/day.

3.3. Free short-chain fatty acid content in fermented milk

3.3.1. Butyric acid

The free short-chain fatty acids (SCFAs) analysed were butyric, caproic and caprylic acids.

The butyric acid (C4) content of pasteurized milk samples fermented at 37 °C for 48 h with different LAB strains varied from a minimum of 9.45 mg 100 g^{-1} in *R(11) to a maximum of 110.50 mg 100 g^{-1} in B (E1) of dry matter (Fig. 2**a-d**). As regard to *Streptococcus* group, among the samples without glutamine addition all samples, except M(96), showed a slight increase in butyric acid concentration compared to the control. In the glutamine-enriched samples, however, there were no statistically significant differences compared to the control, except for T (85)+G. This result could be related to the trend shown in pyroglutamic acid production (Fig. 1**a**). In *Lactobacillus* group shown in Fig. 2**b**, among the samples without added glutamine, *L. bulgaricus* E1 showed the highest concentrations of butyric acid (110.50 mg 100 g^{-1}). The results obtained are in line with Beshkova et al. (1998), who reported butyric acid production in milk fermented with *L. bulgaricus* between 0.86 and 0.91 mg 100 g^{-1} fresh weight. The sample *C(393), inoculated with the strain of *Lcb. casei* ATCC393, showed the highest production of butyric acid (108.02 mg 100 g^{-1} d.m.) compared to the control (10.73 mg 100 g^{-1} d.m.) (Fig. 1**c**). This result is in line with Dimitrellou et al., (2019) and Song et al. (2022) who observed that several strains *Lcb. casei*, produced good quantities of butyric acid. Dimitrellou et al. (2019) and Song et al. (2022) observed that *Lcb. casei* ATCC393 is a good producer of SCFAs, including butyric acid, at the fermented milk and intestinal levels.

In Fig. 1**d**, the microorganisms of the *Lactiplantibacillus* genus are shown. Specifically, the *PL(299) sample exhibits the highest production of butyric acid compared to the control, both with and without glutamine enrichment (32.90 and 27.57 mg 100 g^{-1} d.m., respectively). For the other samples, no statistically significant differences are observed compared to the control. This result is consistent with the observation by Aiello et al. (2023b) of a strong butyrogenic capability of *Lactobacillus plantarum*. According to Beshkova et al. (1998) SCFA production in fermented products shows a strain-specific relationship. The results show that LAB strains belonging to the same species produce significantly different concentrations of butanoic acid. For example, *L. bulgaricus* E1 coded B(E1) showed approximately 500% higher butyric acid production than that of strains B(MY), B(CCB), B(CPV) and B (AZP1), while *Lcb. rhamnosus* strains A3 and A1G coded R(A3) and R (A1G), produced a quantity of butanoic acid 600% and 300% higher than sample *R(11), respectively. After glutamine was added, samples B (E1)+G, *C(393)+G, R(A1G)+G and R(A3)+G showed lower butyric acid content. These results are in line with data reported by Aiello et al. (2023b) who demonstrated that the butyrogenic capacity of different lactic acid bacteria strains is associated with the presence of specific lipases and esterases that are linked to a metabolic pathway involving triglycerides and not amino acids, such as glutamine as substrates. Therefore, adding glutamine to milk could inhibit microbial lipase and esterase production and shift the metabolism of specific strains towards

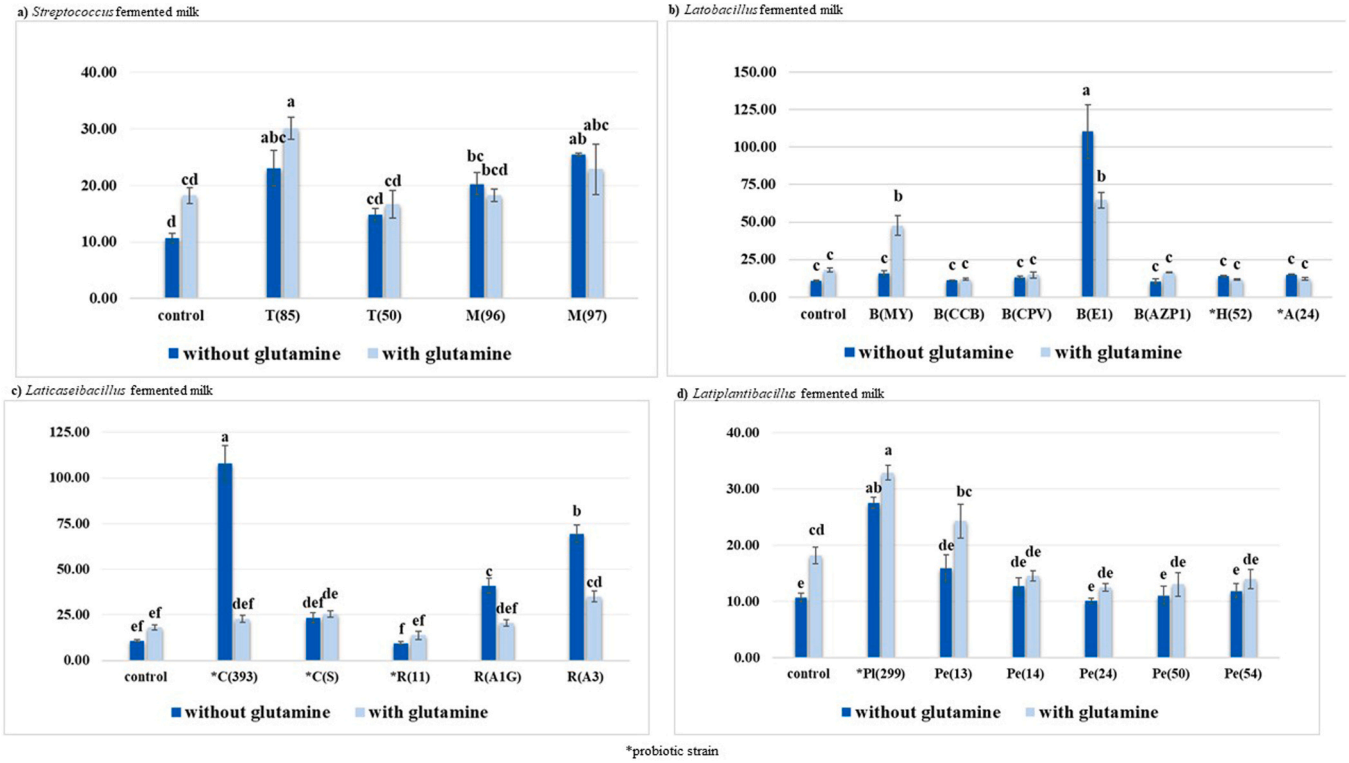

Fig. 2. Butyric acid content (mg 100 g^{-1}d.m.) in fermented milk without and with glutamine (+G) and inoculated with micro-organisms of *Streptococcus* (a), *Lactobacillus* (b), *Lacticaseibacillus*(c) and *Lactiplantibacillus* genus (d). Control sample was the uninoculated milk. **a)** *Streptococcus* fermented milk. *probiotic strain, $^{a\text{-}d}$ different letters on the bars indicate statistically significant differences ($p < 0.05$). **b)** *Lactobacillus* fermented milk. *probiotic strain, $^{a\text{-}d}$ different letters on the bars indicate statistically significant differences ($p < 0.05$). **c)** *Lacticaseibacillus* fermented milk. *probiotic strain, $^{a\text{-}f}$ different letters on the bars indicate statistically significant differences ($p < 0.05$). **d)** *Lactiplantibacillus* fermented milk. *probiotic strain, $^{a\text{-}d}$ different letters on the bars indicate statistically significant differences ($p < 0.05$).

pGlu production rather than C4 production. In the other samples, no statistically significant differences were found in butyric acid production between milk with and without glutamine except for B(MY)+G, as its C4 content was higher than that of the nonenriched substrate. This could be occur because most free fatty acids in milk could originate from amino acids (Beshkova et al., 1998). However, these microbial metabolisms have not been studied to date.

According to Borycka-Kiciak et al. (2017) 200–300 mg daily of butyric acid administered in the form of sodium salt can improve the most common bowel (diarrhoea, irritable bowel syndrome, constipation) and exert an effect in the treatment and prevention of cancer. In addition, (Guilloteau et al., 2010) reported that butyric acid can act as a growth promoter when administered at doses of 0.1–0.5 g/kg in rats.

3.3.2. Caproic and caprylic acid

In addition to C4, the volatile fatty acids caproic (C6) and caprylic (C8) were detected in the fermented milk samples. SCFAs characterize the aroma and flavour of dairy products. C6 in the right quantities generates pungent and floral notes (Pereda et al., 2008), while C8 generates acidity and freshness (Cheng, 2010). As reported in Table 3 (**a-d**), the C6 concentration varies from a minimum of 22.21 mg 100 g^{-1} in PE(24) to a maximum of 83.48 mg 100 g^{-1} in *C(S)+G, while the C8 content varies from a minimum of 15.77 mg 100 g^{-1} in B(CCB)+G to a maximum of 22.28 mg 100 g^{-1} in *C(S)+G. Milk samples inoculated with *St. thermophilus* (85 and 90) and *L. delbruekii subsp. bulgaricus* (MY and E1), represented in Tables 3**a** and 3**b**, showed an increase of approximately 30% in the production of C6 compared to the uninoculated sample, corresponding with reports by various sources in the literature (Dan et al., 2017; Liu et al., 2022). In *Lacticaseibacillus* group (Table 3**c**), the probiotic *Lcb. casei* Shirota, produced higher amounts of C6 and C8 (83.48 mg 100 g^{-1} d.m. and 22.28 mg 100 g^{-1} d.m., respectively) when milk was enriched with glutamine (*C(S)+G). However, the addition of glutamine to milk generally did not affect C6 and C8 production. The probiotic *Lactiplantibacillus plantarum 299 v* (Table 3**d**) also demonstrated good production of C6 and C8, both in the presence and absence of glutamine. As already reported for butyric acid, C6 production showed a strain-dependent relationship (Beshkova et al., 1998). For example, compared to the *R(11) strain of the same species (24.45 mg 100 g^{-1} d.m.), strains A3 and A1G of *Lcb. rhamnosus* showed a higher production of C6 (65.47 and 83.42 mg 100 g^{-1} d.m.).

4. Conclusions

The addition of glutamine influences the ability of lactic acid bacteria to produce pyroglutamic in fermented milk, indicating that enzymes, such as aminopeptidase and glutamine cyclase, are involved in the reaction. Without the addition of glutamine, the amount of pyroglutamic acid increased in fermented milk produced with strains of *Lactobacillus* and *Lactiplantibacillus* genus and the *L. bulgaricus* MY strain produced the greatest amount. Adding glutamine to milk before the inoculation, the amount of pyroglutamic acid further increased also in fermented milk produced with strains of *Streptococcus* and *Lacticaseibacillus* genus, in particular with the strain *L. casei* Shirota.

Among the studied strains *L. bulgaricus* E1 and the probiotic *L. casei* ATCC393 produced the greatest amount of butyric acid in the absence of glutamine. The butyrogenic activity is related to the ability of lactic acid bacteria to produce specific lipases, as previously reported for *L. plantarum*.

Furthermore, short-chain fatty acid production in fermented products showed a strain-specific relationship because strains of lactic acid bacteria in the same species contain different concentrations of free short-chain fatty acids.

Considering the potentially beneficial properties of pyroglutamic and butyric acid and the increasing consumer interest in functional food in the last few years, these results are an interesting starting point for researchers to utilize selected strains of lactic acid bacteria to develop

Table 3
Caproic (C6) and caprylic acid (C8) contents in fermented milk without and with glutamine (+G) and inoculated with micro-organisms of *Streptococcus* (a), *Lactobacillus* (b), *Lacticaseibacillus*(c) and *Lactiplantibacillus* genus (d). Control sample was the uninoculated milk.

a) *streptococcus* fermented milk		
Sample	**C6** (mg 100 g^{-1} d.m.)	**C8** (mg 100 g^{-1} d.m.)
control	24.72 ± 2.03^{d}	16.50 ± 1.19^{c}
control+G	40.25 ± 0.57^{c}	18.53 ± 0.87^{c}
T(85)	52.01 ± 3.15^{abc}	16.68 ± 0.11^{c}
T(85)+G	60.19 ± 2.80^{a}	18.79 ± 0.26^{b}
T(50)	56.85 ± 3.79^{ab}	17.06 ± 0.19^{c}
T(50)+G	42.70 ± 1.54^{bc}	18.32 ± 0.12^{b}
M(96)	41.06 ± 5.25^{c}	16.73 ± 0.72^{c}
M(96)+G	62.30 ± 5.12^{a}	19.22 ± 1.23^{a}
M(97)	60.81 ± 4.74^{a}	16.47 ± 1.14^{c}
M(97)+G	50.73 ± 4.83^{abc}	18.38 ± 0.09^{a}
b) *lactobacillus* fermented milk		
Sample	**C6** (mg 100 g^{-1} d.m.)	**C8** (mg 100 g^{-1} d.m.)
control	24.72 ± 2.03^{d}	16.50 ± 1.19^{abc}
control+G	40.25 ± 0.57^{bc}	18.53 ± 0.87^{abc}
B(MY)	30.48 ± 2.51^{bcd}	16.4 ± 0.33^{abc}
B(MY)+G	64.60 ± 1.17^{a}	18.45 ± 0.46^{a}
B(CCB)	26.46 ± 2.51 ^{cd}	15.77 ± 0.00^{c}
B(CCB)+G	27.28 ± 4.08^{bcd}	17.60 ± 0.12^{acd}
B(CPV)	26.27 ± 2.89 ^{cd}	15.82 ± 0.12^{bc}
B(CPV)+G	33.50 ± 5.31^{bcd}	17.74 ± 0.25^{abc}
B(E1)	41.38 ± 4.95^{b}	17.66 ± 2.19^{abc}
B(E1)+G	60.76 ± 7.55^{a}	18.65 ± 0.41^{ab}
B(AZP1)	24.00 ± 2.09^{d}	15.84 ± 0.00^{bc}
B(AZP1)+G	28.01 ± 0.79^{bcd}	17.56 ± 0.12^{abc}
*H(52)	30.94 ± 1.55^{bcd}	16.34 ± 0.50^{abc}
*H(52)+G	31.77 ± 3.54^{bcd}	17.96 ± 0.61^{abc}
*A(24)	26.20 ± 0.85 ^{cd}	15.84 ± 0.06^{bc}
*A(24)+G	27.40 ± 5.69^{bcd}	17.59 ± 0.11^{abc}
c) *lacticaseibacillus* fermented milk		
Sample	**C6** (mg 100 g^{-1} d.m.)	**C8** (mg 100 g^{-1} d.m.)
control	24.72 ± 2.03 ^{f}	16.50 ± 1.19^{b}
control+G	40.25 ± 0.57^{def}	18.53 ± 0.87^{ab}
*C(393)	41.86 ± 3.14^{def}	16.26 ± 0.40^{b}
*C(393)+G	43.69 ± 3.19^{de}	19.04 ± 1.43^{ab}
*C(S)	47.96 ± 1.73 ^{cd}	16.47 ± 0.31^{b}
*C(S)+G	83.48 ± 3.50^{a}	22.28 ± 2.43^{a}
*R(11)	24.45 ± 3.00 ^{f}	15.88 ± 0.02^{b}
*R(11)+G	25.04 ± 0.75 ^{f}	17.62 ± 0.03^{b}
R(A1G)	65.47 ± 7.59^{b}	16.79 ± 0.24^{b}
R(A1G)+G	29.70 ± 0.44^{ef}	19.08 ± 1.53^{ab}
R(A3)	83.42 ± 11.18^{a}	17.87 ± 0.29^{b}
R(A3)+G	62.56 ± 0.02^{bc}	19.01 ± 0.79^{ab}
d) *Lactiplantibacillus* fermented milk		
Sample	**C6** (mg 100 g^{-1} d.m.)	**C8** (mg 100 g^{-1} d.m.)
control	24.72 ± 2.03^{ef}	16.50 ± 1.19^{cdef}
control+G	40.25 ± 0.57^{c}	18.53 ± 0.87^{abc}
*Pl(299)	50.54 ± 1.08^{b}	17.13 ± 0.01^{bcdef}
*Pl(299)+G	62.87 ± 1.23^{a}	19.39 ± 0.51^{a}
Pe(13)	32.36 ± 1.82^{cdef}	16.25 ± 0.00^{def}
Pe(13)+G	32.24 ± 0.88^{cdef}	17.89 ± 0.40^{abcde}
Pe(14)	27.42 ± 3.40^{def}	16.16 ± 0.27^{def}
Pe(14)+G	31.51 ± 3.07^{cdef}	18.61 ± 0.76^{ab}
Pe(24)	22.21 ± 0.93 ^{f}	15.78 ± 0.13 ^{f}
Pe(24)+G	25.34 ±0.99^{def}	17.63 ± 0.06^{abcdef}
Pe(50)	27.39 ± 3.24^{def}	15.96 ± 0.17^{ef}
Pe(50)+G	32.56 ± 4.46^{cde}	18.24 ± 0.55^{abcd}
Pe(54)	26.95 ± 3.61^{def}	15.90 ± 0.04^{ef}
Pe(54)+G	35.48 ± 3.79 ^{cd}	18.43 ± 0.46^{abc}

$^{a-d}$different superscript letters in the same column indicate statistically significant differences ($p < 0.05$).
*probiotic strain, $^{a-d}$different superscript letters in the same column indicate statistically significant differences ($p < 0.05$).
*probiotic strain, $^{a-f}$different superscript letters in the same column indicate statistically significant differences ($p < 0.05$).
*probiotic strain, $^{a-f}$different superscript letters in the same column indicate statistically significant differences ($p < 0.05$).

new dairy products with functional properties.

CRediT authorship contribution statement

Giuseppe Blaiotta: Validation, Supervision, Resources. **Martina Calabrese:** Writing – original draft, Formal analysis. **Fabiana PIZZOLONGO:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Lucia De Luca:** Software, Investigation, Data curation. **Raffaele Romano:** Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Maria Aponte:** Validation, Supervision, Resources.

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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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2024.106175.

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