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Preliminary study on kinetics of pyroglutamic acid formation in fermented milk

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ABSTRACT

Pyroglutamic acid (pGlu) influences the aromatic and sensory properties of foods and has several benefits for human health. The presence and kinetics of pGlu formation in fermented milk samples were investigated from a chemical point of view. Plain yoghurt, kefir and other probiotic fermented milk products available on the market were analysed to quantify lactic acid and pGlu. The pGlu concentrations in fermented milks ranged from 51.65 to 277.37 mg 100 g^{-1} dry matter. Laboratory-scale fermented milk was produced, and samples were taken at different times of fermentation and storage to construct the kinetics curve. At the beginning of the fermentation process, pGlu was already present in UHT milk (188.69 mg 100 g^{-1} dry matter) used to elaborate fermented milk, and its content increased not only during fermentation but during storage as well, reaching up to 403.56 mg 100 g^{-1} dry matter after 30 days.

1. Introduction

Pyroglutamic acid (pGlu), also known as pidolic acid or 5-oxoproline, is a bioactive glutamine derivative that plays a key role in preserving the quality and nutritional value of foods. It is a five-membered lactam that is formed from glutamic acid through enzymatic and nonenzymatic pathways. The amino group of glutamic acid or glutamine cyclises into a γ-lactam ring by deamidation or dehydration, with a spontaneous or thermal-assisted nucleophilic reaction of the α-amino group with the γ-carboxyl group (Kumar & Bachhawat, 2012). Previous studies indicate that pGlu has pharmacological properties and has antimicrobial, antitumoural (Kimura, 2005; Kimura, Kido, Takaku, Sumiyoshi, & Baba, 2004), mitogenic (Inoue et al., 2015; Oono et al., 2009), anxiolytic (Antonelli, Carla, Lambertini, Moroni, & Bianchi, 1984; Sinforiani et al., 1985), antidiabetic and hypolipidaemic activities (Yoshinari & Igarashi, 2011).

Pyroglutamic acid is present as the terminal amino acid in several biologically significant peptides and proteins, such as hormones and neuropeptides (Kumar & Bachhawat, 2012), and as a free form in the epidermis (Solano, 2020), brain (Forgacsova et al., 2018), eye (Jiang, Yang, Zheng, Liu, & Chen, 2020), plasma, and cerebrospinal fluids (Eckstein, Ammerman, Reveles, & Ackermann, 2008).

In food, pGlu is found both as an amino residue in proteins or pyroglutamyl peptides and in the free form. Factors such as heat, high pressure, enzymatic modifications or combinations of these factors contribute to free pyroglutamic acid formation during food processing (Kiyono et al., 2013; Kumar & Bachhawat, 2012). Pyroglutamyl peptides have been found in wheat gluten and potato hydrolysates (Higaki-Sato et al., 2003; Yao & Udenigwe, 2018), acid-digested edible mushrooms such as *Agaricus campestris* (Gazme, Boachie, Tsopmo, & Udenigwe, 2019), Japanese rice wine (Kiyono et al., 2013) and dry-cured ham (Paolella et al., 2018). Sforza Cavatorta, Galaverna, Dossena, and Marchelli (2009) identified pyroglutamyl-amino acids in Parmigiano Reggiano cheese and found that the accumulation of this molecule, together with γ-glutamyl- and lactoyl-amino acids, can be usefully exploited to estimate the actual age of Parmigiano Reggiano. Moreover, their formation sequesters bitter amino acids (Phe, Leu, Ile, Val) from the amino acidic pool, transforming them into derivatives that have been demonstrated to have a more "umami" taste.

The presence of free pGlu has been reported in canned tomato juice, ranging from 967 mg kg^{-1} to 2681 mg kg^{-1} (Marconi, Floridi, & Montanari, 2007), and in donkey milk (456 mg L^{-1}) and formula milk (111 mg L^{-1}) (Murgia et al., 2016). pGlu is also present in high amounts (0.5 g 100 g^{-1}) in many cheese varieties, particularly in aged Italian cheeses such as Grana Padano and Parmigiano Reggiano

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(Mucchetti et al., 2000) since it is a parameter reasonably related to the ripening time (Masotti, Hogenboom, Rosi, De Noni, & Pellegrino, 2010). In fact, Mucchetti et al. (2000) showed a correlation between cheese age (from 2 to 24 months) and free pGlu concentration that increases because of the progressive cyclisation of glutamine.

In fermented food, such as ripened cheese (Mucchetti et al., 2000) and Zimbabwean naturally fermented milk (Gadaga, Mutukumira, & Narvhus, 2001), it has also been hypothesised that pGlu production might depend on the starter microflora rather than on the substrate because of glutamine cyclase and pyrrolidone carboxyl peptidase activities. These enzymes are released by microbial cultures, such as *Lactobacillus helveticus, Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lb. delbrueckii* subsp. *lactis* and *Streptococcus thermophilus* (Mucchetti et al., 2000; Mucchetti, Locci, Massara, Vitale, & Neviani, 2002). Murgia, Scano, Cacciabue, Dessì, and Caboni (2019) found pGlu in yoghurts from sheep and goat milk. They found that pGlu and β-phenyllactic acid levels are higher in goat yoghurt than in sheep yoghurt. Furthermore, Pinto et al. (2020) found pGlu in probiotic and synbiotic yoghurt manufactured with *Lactobacillus acidophilus* and *Bifidobacteria* strains without inulin or fortified with 1 and 3% (w/w) inulin.

However, to the best of our knowledge, this research area has been scarcely investigated, and studies that discuss the presence and kinetics of pGlu formation in fermented milk products, such as yoghurt, are still lacking. Since pGlu could have beneficial effects on human health, this work focused on quantifying pGlu in different fermented milk types, i.e., yoghurt, kefir and other probiotic fermented milk products available on the market, and defining the kinetics of pGlu formation to increase knowledge on the chemical composition of fermented milk.

2. Materials and methods

2.1. Materials

Different commercially available yoghurt and fermented milk types were purchased from a local market and stored at −18 °C until analysis. Samples were as follows: LFY1−LFY3, low fat plain yoghurts; WY1−WY3, whole fat plain yoghurts; LFGY1 and LFGY2, Greek low fat plain yoghurts; WGY1 and WGY2, Greek whole fat plain yoghurts; PFM1−PFM5, probiotic fermented milk; FFM1 and FFM2, functional ferment milk (with 1.6% phytosterols); K1−K3, kefir. The category, type, protein percentage, fat percentage and microbial composition reported on the label of each product are shown in Table 1. Three production batches for each type of sample were purchased.

Laboratory-scale yoghurt was produced in triplicate using 1000 mL of commercial ultra high temperature (UHT) sterilised whole milk (3.4% protein, 3.6% fat and 5% lactose) and inoculating with a blend of *Str. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* (1:1 ratio, Yoghurt Linea, supplied from INRAO s.r.l., Milano, Italy) at a rate of 2% (w/w). The incubation was carried out at 40 °C in an incubator (Panasonic MIR-154-PE Cooled Incubator, Osaka, Japan) until milk clotting occurred after 5 h (when the initial pH value of 6.6 had dropped to 4.3). A sample of UHT-sterilised whole milk without addition of microorganisms was incubated under the same conditions for 5 h and used as a control. Then, the set yoghurt and milk control samples were placed into a cold room (4 ± 1 °C) and stored for 30 days. The samples were analysed at 2-day intervals during cold storage.

2.2. Chemicals

All solvents and reagents used in determinations were purchased from the Sigma−Aldrich Co. (Milano, Italy).

2.3. pH determination

Determination of pH, representing the hydrogen ion concentrations in yoghurt and fermented milk, was carried out by a pH meter (Crison Basic 20, Barcelona, Spain).

2.4. Moisture content

Representative samples (~3 g each) of the yoghurt and fermented milk were dried at 102 °C for 2 h in an air oven (Thermo Electron corporation, Waltham, MA, US) so that the moisture and dry matter (dm) could be determined gravimetrically (Koc, Yilmazer, Balkır, & Ertekin, 2010).

Table 1
Commercial specifications (category, type, protein and fat content) and microbial compositions of samples of yoghurt, probiotic fermented milk, functional fermented milk and kefir used in this study.[a]

Samples	Category	Type	Protein (%)	Fat (%)	Microbial composition
LFY1	Plain yoghurt	Low-fat	6	0	*Str. thermophilus; Lb. bulgaricus*
LFY2			5	0	*Str. thermophilus; Lb. bulgaricus*
LFY3			4	0	*Str. thermophilus; Lb. bulgaricus*
WY1		Whole	4.8	3.7	*Str. thermophilus; Lb. bulgaricus*
WY2			3.7	4.2	*Str. thermophilus; Lb. bulgaricus*
WY3			3	3.4	*Str. thermophilus; Lb. bulgaricus*
LFGY1	Plain Greek	Low-fat	10	0	*Str. thermophilus; Lb. bulgaricus*
LFGY2	yoghurt		10	0	*Str. thermophilus; Lb. bulgaricus*
WGY1		Whole	7.8	5	*Str. thermophilus; Lb. bulgaricus*
WGY2			9	5	*Str. thermophilus; Lb. bulgaricus*
PFM1	Fermented milk	Probiotic	1	0	*Lb. casei* Shirota
PFM2			3.9	3.4	*Str. thermophilus; Lb. bulgaricus; Bifidobacterium* spp.
PFM3			3	1.6	*Lb. paracasei* subsp. *paracasei* CNCM I-3689; *Str. thermophilus;Lb. bulgaricus*
PFM4			2.9	0.9	*Lb. casei* Activ.
PFM5			2.4	0.9	*Str. thermophilus; Lb. johnsonii* La1
FFM1		Functional (with 1.6%	3.2	1.1	*Str. thermophilus; Lb. bulgaricus*
FFM2		phytosterols)	2.7	1.5	*Lb acidophilus; Bifidobacterium* spp.
K1		Kefir	3.7	6.8	*Lc. lactis; Lc. cremoris;Lb. acidophilus; Lb. helveticus;Lb. lactis; B. lactis; S. thermophilus*
K2			3.7	6.8	*Lc. lactis; Lc. cremoris;Lb. acidophilus; Lb. helveticus;Lb. lactis B. lactis; Str. thermophilus*
K3			3.5	1.5	*Lc. lactis; Lc. cremoris; Lb. acidophilus; Lb. helveticus;Lb. lactis; B. lactis; Str. thermophilus*

[a] Abbreviations are: LFY, low-fat yoghurt; WY, whole yoghurt; LFGY, low-fat Greek yoghurt; WGY, whole Greek yoghurt; PFM, probiotic fermented milk; FFM functional fermented milk; K, kefir; *Str.*, *Streptococcus*; *Lb.*, *Lactobacillus*; *Lc.*, *Lactococcus*; *B.*, *Bifidobacterium*.

2.5. HPLC determination of sugar content

Sugar extraction was performed by dissolving one gram of yoghurt sample in 10 mL of 0.5 M sulphuric acid, and centrifuging at 10,000×*g* for 10 min (Wang et al., 2010). The supernatant was collected and filtered with a 0.45 μm filter.

The glucose, galactose and lactose contents were determined by injecting 20 μL supernatant into an HPLC (Agilent 1100; Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a refractive index detector (G1362A). The isocratic mobile phase was water/acetonitrile (25:75, v/v), the flow rate was set at 1.7 mL min^{-1}, and a ZORBAX carbohydrate NH_2 column (4.6 × 250 mm, 5 μm; Agilent Technologies Inc.) was used. The calibration curves were constructed with glucose, galactose and lactose standard solutions (2500, 5000, 10,000 ppm) in water/acetonitrile (25:75, v/v). All standard solutions and extracted samples were injected in triplicate; the results were expressed as percentages (%) (g 100 mL^{-1} sample).

2.6. HPLC determination of pyroglutamic acid and lactic acid content

Pyroglutamic and lactic acid extraction was carried out according to the method for polar acids described by Bevilacqua and Califano (1989), with some modifications. Approximately 3.5 g of each sample was added to 25 mL 0.5% (w/w) $(NH_4)_2HPO_4$ in bidistilled water, stirred for 1 min, extracted for 1 h, and then centrifuged at 7500×*g* for 10 min in a multispeed centrifuge (PK 131, ALC International Srl, Milano, Italy). Supernatant was filtered through filter paper and a 0.45-μm PES hydrophilic membrane filter. Triplicate extractions were performed for all samples.

Pyroglutamic and lactic acids were quantified by high performance liquid chromatography (HPLC) according to the method reported by Marconi et al. (2007), with some modifications. Briefly, 20 μL of each extract was injected into an Agilent 1100 series HPLC equipped with a quaternary pump, G4225A degasser, DAD G1315B and FLD G122A detectors and an Eclipse XDB-C18 reversed-phase column (150 mm × 4.6 mm, 50 μm; Agilent Technologies). 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. Detector wavelength was set at 210 nm. Calibration curves for DL − pyroglutamic acid and lactic acid were constructed with the standard solution (1, 10, 50, 100, 1000 ppm) in bidistilled water. Accuracies of the methods used to determine the pGlu and lactic acid contents were 91.2 ± 2.2% and 90.8 ± 1.3%, respectively. The limits of detection (LODs) and quantification (LOQs) of the method used for pGlu were 1 and 3 ppm, respectively. The LOD and LOQ of the method for lactic acid were 50 and 150 ppm, respectively.

All standard solutions and extracted samples were injected in triplicate; the results were expressed as percentages (%) (g 100 g^{-1} of dry matter) for lactic acid and as mg 100 g^{-1} of dry matter for pGlu acid. A typical chromatogram is shown in Fig. 1, where the most abundant peaks were pGlu and lactic acid.

2.7. Statistical analysis

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

3. Results and discussion

3.1. pH and lactic acid content of commercial yoghurt and fermented milk

Acidity is one of the major indices for consumers' acceptability of plain yoghurt because acid and flavour development are closely related in this fermented product. Usually high acidity is not appreciated by consumers. Acid development may be monitored by measuring the pH and the lactic acid content.

Fermented foods naturally exhibit low pH values due to the transformation of fermentable sugars into organic acids by starter microorganisms; therefore, a high organic acid concentration in the substrate is positively correlated with low pH because of acid dissociation in an aqueous medium (Casolari, 2007). The commercial samples analysed showed average pH values from 3.47 to

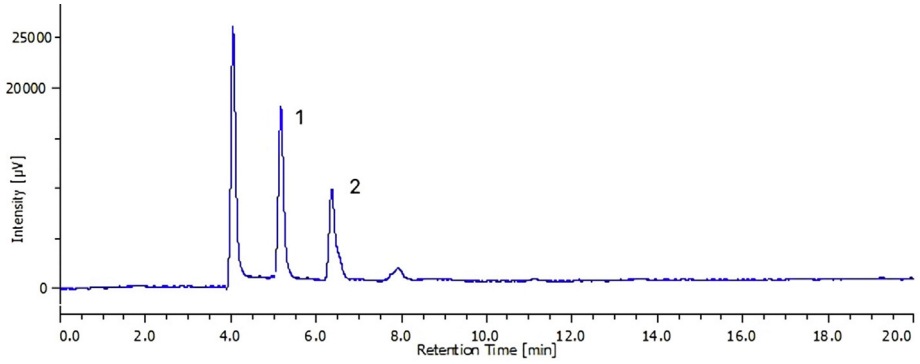

Fig. 1. Typical HPLC chromatogram of lactic (1) and pyroglutamic (2) acids.

4.47 in the fermented probiotic milk PFM1 and the whole Greek yoghurt WGY1, respectively (Table 2), in line with the range of 4.00–4.60 reported by different literature sources (Al-Kadamany, Khattar, Haddad, & Toufeili, 2003; Aryana & McGrew, 2007). In particular, Aryana and McGrew (2007) found mean pH values ranging from 4.32 to 4.60 in yoghurt with *Lactobacillus casei* and various prebiotics; Al-Kadamany et al. (2003) found a pH range of 3.47–4.05 in concentrated yoghurt during shelf life. Wang et al. (2010) found that pH values of yoghurts inoculated with *Lb. casei* Zhang during storage at 4 °C for up to 21 days decreased from 4.20 to 3.43.

Lactic acid represents the main fermentation product from lactose, which gives yoghurt a sharp and acidic taste. The concentration of lactic acid varies from 0.04 to 0.3% in fermented milk (Aiello, Pizzolongo, Manzo, & Romano, 2019) to 0.8–1.3% in yoghurt (Liu & Lv, 2019), mainly depending on the microbial cultures and the conditions of time and temperature of the fermentation (Abdel-Rahman, Tahisro, & Sonomoto, 2013). Lactic acid content can affect the taste (acid or refreshing) and shelf life of fermented milk, preventing development of putrefactive bacteria. Moreover, this organic acid has significant impacts on the digestibility of caseins, on the absorption of mineral salts and on pH and intestinal regularity (Salvadori del Prato, 2005). In the samples analysed (Table 2), the lactic acid contents were found to range from 3.50% to 8.52% (in FFM2 and LFY3, respectively), similar to the concentrations usually found in this fermented product category. In fact, in sweetened and natural yoghurts, Vénica, Perotti, and Bergamini (2014) found lactic acid concentrations ranging from 5.75 to 6.25% at the end of production, and from 6.85 to 7.86% after 28 days of storage at 5 °C. Regarding the lactic acid concentration in kefir, Guzel-Seydim, Seydim, and Green (2000) showed a lower concentration that ranged from 4.57% at time 0 of storage to 5.50% after 21 days of storage, while Gronnevik, Falstad, and Narvhus (2011) found a concentration of lactic acid of 5.71% after 8 weeks of storage at 5.5–6 °C.

3.2. pGlu content in commercial yoghurt and fermented milk

Table 2 shows the pGlu contents in all samples of each category of fermented milk. LFY3 showed the highest pyroglutamic content (277.37 mg 100 g^{-1} dm), while PFM1 had the lowest (51.65 mg 100 g^{-1} dm). Low-fat yoghurt (LFY) exhibited the highest content of pyroglutamic acid (200.22–277.33 mg 100 g^{-1} dm), while whole Greek yoghurt (WGY) showed the lowest concentration (106.14–146.04 mg 100 g^{-1} dm).

No statistically significant differences were found among the categories of functional fermented milk (FFM), probiotic fermented milk (PFM), low-fat Greek yoghurt (LFGY), whole plain yoghurt (WY) and kefir (K). In fact, the average concentrations of pGlu were 172.06 mg 100 g^{-1} dm for WY, 139.94 mg 100 g^{-1} dm for LFGY, 135.23 mg 100 g^{-1} dm for PFM, 133.06 mg 100 g^{-1} dm for FFM and 193.63 mg 100 g^{-1} dm for K samples.

The presence of pGlu in fermented milk and yoghurt may depend on the cyclase activity of thermophilic lactic acid bacteria (especially *Str.s thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*) as reported by Mucchetti et al. (2002). However, Mucchetti et al. (2000) showed a correlation between ripening time and pGlu content, which ranged from 354 to 722 mg 100 g^{-1}, in Grana Padano cheese. Additionally, Ochi et al. (2013) showed that pGlu could be used as a marker of the ripening process of Cheddar cheese. Therefore, since the storage period of fermented milk is shorter than the ripening time of the cheeses, pGlu concentrations in fermented milk (on average 21.34 mg 100 g^{-1}) are lower than those of aged cheeses.

Indeed, Careri, Spagnoli, Panari, Zannoni, and Barbieri (1996) showed a concentration of pGlu in Parmigiano Reggiano cheese ripened for 24 months with a mean of 485 mg 100 g^{-1} of cheese. Furthermore, Olsen, Vhile, Porcellato, Kidane, and Skeie (2021) showed the influence of cow feeds on pGlu concentration in Gouda cheese ripened for 15 weeks, which ranged from 12.39 mg 100 g^{-1} in cheese obtained from the milk of cows fed barley to 14.19 mg 100 g^{-1} in cheese obtained from the milk of cows fed soybean.

3.3. Assessments of pH, lactic acid, moisture and sugars in laboratory-scale yoghurt

Usually, in the manufacture of yoghurt, the heated milk is conventionally sterilised at 82–90 °C for 5–30 min to guarantee the total elimination of the altering bacterial flora and spores that could be present and could compromise the development of the starter cultures. Furthermore, sterilised milk is particularly suitable

Table 2
Values of pH, moisture, lactic acid and pyroglutamic acid (pGlu) content in commercial yoghurt and fermented milk.[a]

Sample	pH	Moisture (%)	Lactic acid (% dm)	pGlu (mg 100 g^{-1} dm)
LFY1	4.17 ± 0.01^{bcd}	81.38 ± 0.11^{fghi}	6.28 ± 0.11^{cdef}	200.22 ± 25.48^{abcd}
LFY2	4.15 ± 0.01^{bcd}	86.24 ± 0.34^{bcd}	7.12 ± 0.21^{abcd}	260.17 ± 31.51^{ab}
LFY3	4.13 ± 0.01^{bcde}	88.21 ± 0.25^{abc}	8.52 ± 0.12^{a}	277.37 ± 48.68^{a}
WY1	4.17 ± 0.01^{bcd}	83.93 ± 0.17^{def}	4.16 ± 0.36^{hi}	192.23 ± 32.74^{bcd}
WY2	4.03 ± 0.01^{ef}	85.31 ± 0.34^{cde}	5.14 ± 0.29^{fgh}	175.37 ± 33.34^{cde}
WY3	3.85 ± 0.01^{g}	78.95 ± 0.68^{i}	5.50 ± 0.67^{efgh}	148.56 ± 20.99^{cde}
LFGY1	4.20 ± 0.02^{bc}	82.04 ± 1.04^{fgh}	5.25 ± 0.26^{fgh}	142.95 ± 32.91^{de}
LFGY2	4.08 ± 0.02^{def}	84.23 ± 0.20^{def}	7.89 ± 0.60^{ab}	136.92 ± 8.54^{de}
WGY1	4.47 ± 0.01^{a}	79.15 ± 0.01^{hi}	6.98 ± 0.19^{bcde}	146.04 ± 8.27^{cde}
WGY2	4.11 ± 0.01^{cdef}	81.25 ± 0.48^{fghi}	4.138 ± 0.68^{hi}	106.14 ± 30.48^{ef}
PFM1	3.47 ± 0.01^{h}	85.53 ± 0.89^{cd}	4.07 ± 0.96^{hi}	51.65 ± 12.80^{f}
PFM2	4.14 ± 0.01^{bcd}	86.52 ± 0.23^{abcd}	4.95 ± 0.34^{fghi}	222.57 ± 79.56^{abc}
PFM3	4.22 ± 0.02^{b}	81.87 ± 0.87^{fghi}	4.07 ± 0.14^{hi}	153.73 ± 13.43^{cde}
PFM4	3.90 ± 0.01^{g}	82.44 ± 0.01^{efg}	4.58 ± 0.31^{ghi}	103.10 ± 30.28^{ef}
PFM5	4.12 ± 0.01^{bcde}	83.66 ± 0.67^{def}	6.30 ± 0.23^{cdef}	145.09 ± 9.35^{de}
FFM1	4.18 ± 0.01^{bcd}	85.53 ± 0.73^{ab}	4.65 ± 0.18^{ghi}	152.28 ± 12.79^{cde}
FFM2	4.01 ± 0.01^{f}	80.23 ± 0.08^{ghi}	3.50 ± 0.09^{i}	126.97 ± 27.47^{ef}
K1	4.16 ± 0.01^{bcd}	83.61 ± 0.07^{def}	5.84 ± 0.65^{defg}	161.09 ± 9.39^{cde}
K2	4.20 ± 0.01^{bc}	84.06 ± 2.11^{def}	6.04 ± 0.27^{cdefg}	166.52 ± 20.48^{cde}
K3	4.09 ± 0.10^{def}	89.50 ± 0.34^{a}	7.51 ± 0.87^{abc}	253.29 ± 15.63^{ab}

[a] Abbreviations are: LFY, low-fat yoghurt; WY, whole yoghurt; LFGY, low-fat Greek yoghurt; WGY, whole Greek yoghurt; PFM, probiotic fermented milk; FFM functional fermented milk; K, kefir; dm, dry matter. Different superscript letters in the same column indicate statistically significant differences ($p < 0.05$).

due to the formation of insoluble adducts of β-lactoglobulin and κ-casein at high temperatures (Verruck, Sartor, & Marenda, 2019) and formation of a stable clot that is not subject to syneresis (Alais, 2000).

The milk used in this work was UHT-sterilised, as several reports have shown that UHT has many possible advantages over the conventional method of sterilisation, including better process control and sanitation, energy and time savings, high microbial quality, a longer shelf life of the product and the stimulation of growth and activity of yoghurt cultures (Krasaekoopt, Bhandari, & Deeth, 2003).

Yoghurt formation occurred 5 h after addition of the microorganisms, that is, when the pH of milk changed from the initial value of 6.6 to 4.4 (Fig. 2a). During this time, the pH in the milk control changed to 6.4 and did not produce any clots. The reduction in pH, as observed by Adamberg, Kask, Laht, and Paalme (2003), is linked to the increase in lactic acid, which ranged from 0.38 to 4.33% in the dm (Fig. 2a). The pH and lactic acid trend in yoghurt during 30 days of storage is shown in Fig. 2b. The pH value changed from 4.4 to 4.2 and the lactic acid content increased from 4.33 to 6.03%.

The moisture content of yoghurt was 87.90%. Mahmood, Abbas, and Gilani (2008) showed that the moisture content in plain yoghurt prepared with buffalo milk was 80.47%; Curti, Vidal, Curti, and Ramón (2017) showed a concentration of 82.60% in yoghurt prepared with ultrapasteurised milk, while Sahan, Yasar, and Hayaloglu (2008) showed a concentration that ranged from 86.62% to 87.21% of moisture in no-fat yoghurts prepared with different contents of β-glucans. Furthermore, Vénica et al. (2014) showed a moisture content of 88% for natural yoghurt and 82% for sweetened yoghurt.

To characterise the qualitative and quantitative changes in sugar content during fermentation at 40 °C, lactose, galactose and glucose were analysed in laboratory samples. The lactose content of the milk at the beginning of fermentation was 4.9% and remained constant throughout the 5 h incubation at 40 °C and the 30-day storage at 4 °C in uninoculated milk (Fig. 3). In the inoculated

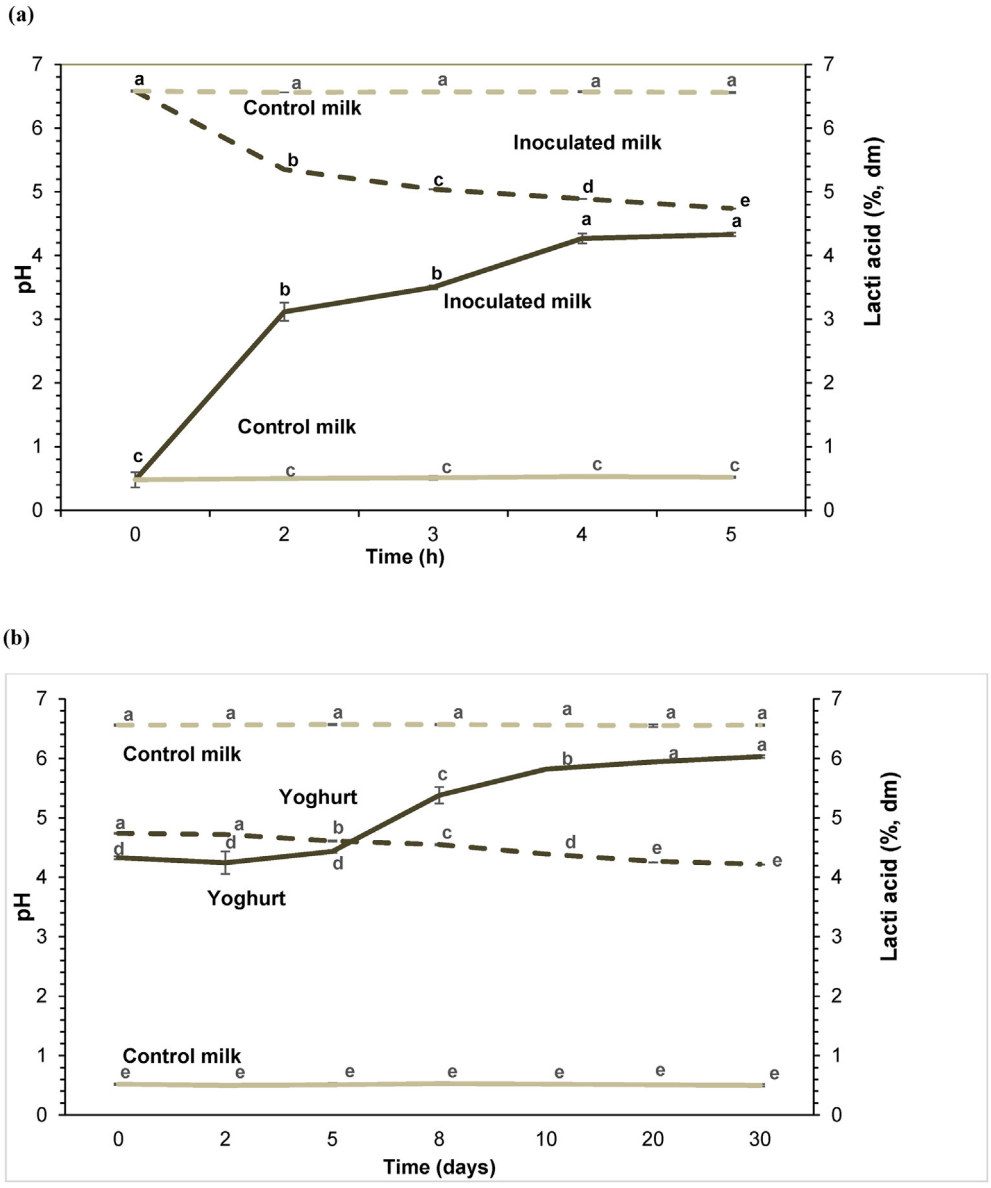

Fig. 2. pH (dashed lines) and lactic acid (solid lines; % dry matter) during (a) the 5-h incubation at 40 °C in inoculated milk (dark lines) and (b) storage at 4 °C in yoghurt (dark lines) compared with the uninoculated milk (light lines; control). Different letters on the same line indicate statistically significant differences (p value < 0.05).

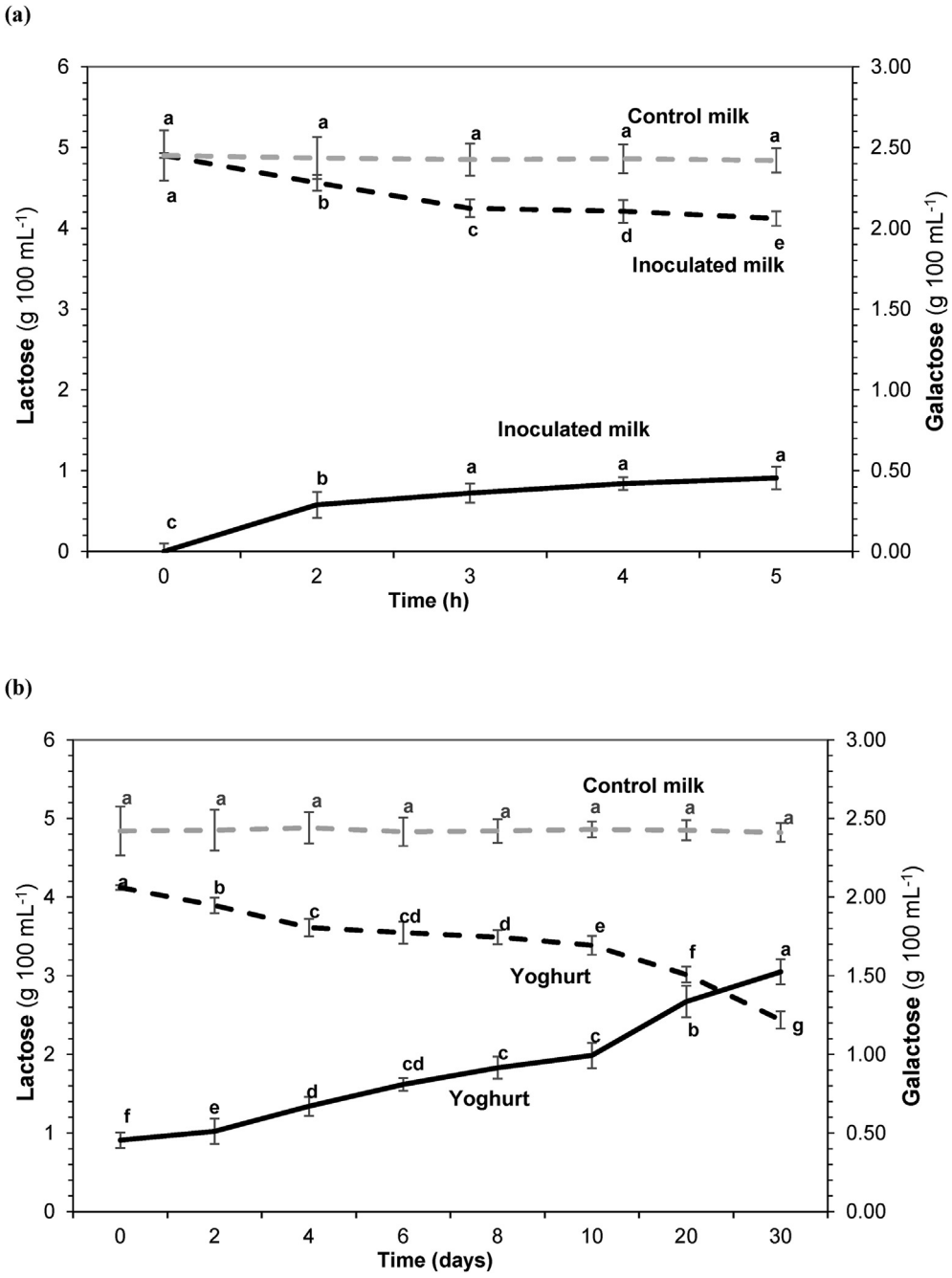

Fig. 3. Lactose (dashed lines) and galactose (solid lines) (g 100 mL^{-1}) during (a) the 5-h incubation at 40 °C in inoculated milk (dark lines) and (b) storage at 4 °C in yoghurt (dark lines) compared with the uninoculated milk (light lines; control). Different letters on the same line indicate statistically significant differences ($p < 0.05$).

milk, the lactose content decreased during fermentation to 4.1% (decrease of approximately 16%) after 5 h, when clot formation occurred (Fig. 3a) and to 2.4% (final decrease of 39%) after 30 days of storage (Fig. 3b). The galactose content was not detected (<0.05%) in the milk before incubation but increased to 0.45% during fermentation at 40 °C and to 1.5% during 30 days of storage at 4 °C (Fig. 3). Galactose and glucose are derived from lactose hydrolysis by microorganisms. These results are in agreement with those of Wang et al. (2010), who found that lactose content decreased to 2.1% in yoghurt inoculated with *Lb. casei* Zhang after 21 days of storage and that galactose increased. The results are also in agreement with those of Delgado-Fernández, Corzo, Olano, Hernández-Hernández, and Moreno (2019) and Delgado-Fernández et al. (2020) who similarly found a final decrease of 38–43% in lactose after 28 days of storage of yoghurts. Glucose was not detected (<0.05%) throughout fermentation and storage, probably due to the preferential metabolism of glucose by the microbial culture.

3.4. Pyroglutamic acid formation in laboratory scale yoghurt

Fig. 4a shows the kinetics of pGlu formation in milk during fermentation at 40 °C to produce yoghurt and in the uninoculated control milk. Surprisingly, pGlu was already present in UHT sterilised milk at the beginning of fermentation (188.47 mg 100 g^{-1} dm). This could be due to the spontaneous conversion of

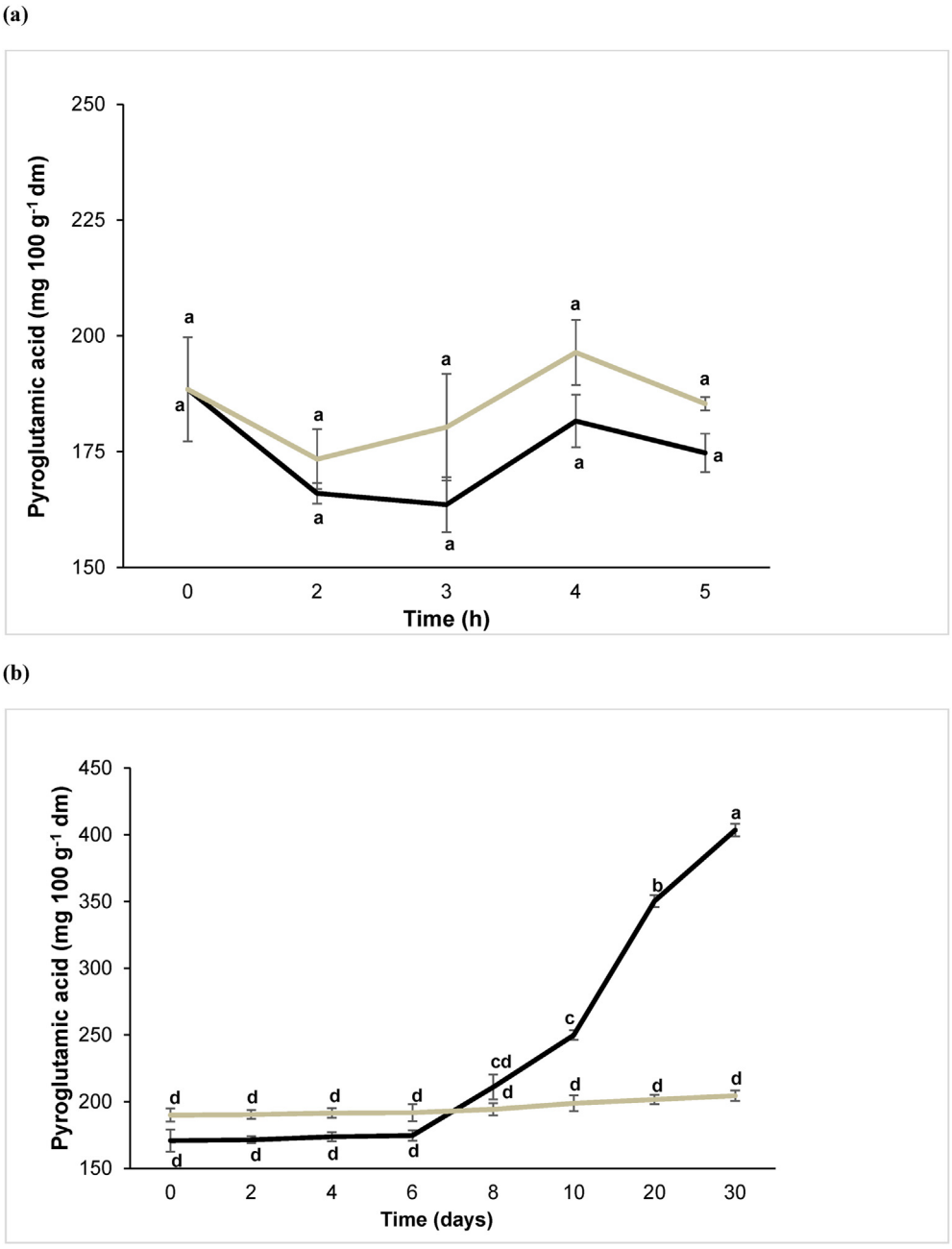

Fig. 4. Kinetics of pyroglutamic acid formation (mg 100 g^{-1} dry matter) during (a) the 5-h incubation at 40 °C in inoculated milk (dark lines) and (b) storage at 4 °C in yoghurt (dark lines) compared with the uninoculated milk (light lines; control). Different letters on the same line indicate statistically significant differences ($p < 0.05$).

glutamine into pGlu by the loss of a water molecule at high temperature (Kumar & Bachhawat, 2012) during the sterilisation treatment carried out on the milk. After 5 h fermentation, pGlu slightly decreased (from 188.47 to 174.74 mg 100 g^{-1} dm) but not in a statistically significant way (Fig. 4a). The same trend was observed by Mugula, Nnko, Narvhus, and Sørhaug (2003), who noted a reduction in pGlu during fermentation by studying the organic acid content in togwa (a Tanzanian fermented food). This decrease in pGlu content observed during fermentation probably occurred due to the chemical balance between glutamine and the lactam derivative in aqueous medium.

During cold storage at 4 °C in yoghurt, the previously produced pGlu concentration gradually increased in the first six days and then quickly increased to 403.56 mg 100 g^{-1} dm at 30 days (Fig. 4b). The pGlu concentration was constant at 190.10–200.96 mg 100 g^{-1} dm in the control throughout the 30 days. Despite the natural pGlu content of the starting substrate, a total increase of more than double was observed in pGlu content at 30 days of cold storage compared with the control. This increase is related to the starter microflora rather than to the raw milk. Indeed, it is known that yoghurt bacteria (*Str. thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus*) release high amounts of free amino acids and show high aminopeptidase and dipeptidyl activity (Shihata & Shah, 2000). Free glutamine and glutamate are then cyclised by microbial enzymes released in the medium by cell lysis, as reported by Mucchetti et al. (2002) for hard-cooked cheeses and Grana Padano cheese and by Liu, Chen, and Lin (2002) for a traditional Chinese fermented rice product. In particular, enzymes responsible for this cyclisation could be glutamate 5-kinase, identified in *Str. thermophilus* by Massarelli, Forlani, Ricca, and De Felice (2000), that

catalyses the phosphorylation of glutamate which becomes highly unstable and prone to spontaneous cyclisation into pyroglutamic acid (Kumar & Bachhawat, 2012), or glutamine cyclase, which is present in both *Str. thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus,* as reported by Mucchetti et al. (2002).

4. Conclusions

The quantitative determination of pyroglutamic acid in commercially available fermented milk with different protein or fat contents allowed us to generate a database that is currently absent in the literature. The tested samples of yoghurt, kefir and other probiotic fermented milk contained pGlu ranging from 51.65 to 277.37 mg 100 g^{-1} dm as a function of the thermophilic lactic acid bacteria used as starter cultures.

When using *Str. thermophilus* and *Lb. bulgaricus* to produce laboratory-scale yoghurt, it was observed that, surprisingly, pyroglutamic acid was already present (188.47 mg 100 g^{-1} dm) in the milk used to produce yoghurt at the beginning of fermentation. During the 5 h fermentation process undertaken to produce yoghurt (until a pH of 4.3 was reached), this content was constant but strongly increased to 403.56 mg 100 g^{-1} dm after 30 days of cold storage.

These findings are also interesting because pGlu in food could have beneficial effects (antitumoural, mitogenic, anxiolytic, antidiabetic and hypolipidaemic activities) on human health. However, further in vitro and in vivo studies are necessary to demonstrate the bioaccessibility and bioavailability of pGlu in fermented milk.

Author contributions

Conceptualization, Raffaele Romano and Fabiana Pizzolongo; methodology, Fabiana Pizzolongo; validation, Alessandra Aiello and Lucia De Luca; formal analysis, Alessandra Aiello; investigation, Alessandra Aiello and Emanuela Pepe; data curation, Alessandra Aiello; writing—original draft preparation, Alessandra Aiello; writing—review and editing, Fabiana Pizzolongo; visualization, Raffaele Romano, Alessandra Aiello and Lucia De Luca; supervision, Fabiana Pizzolongo; project administration, Raffaele Romano; funding acquisition, Raffaele Romano. All authors have read and agreed to the published version of the manuscript.

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.

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