# UNIVERSITY OF NAPLES FEDERICO II

# DEPARTMENT OF AGRICULTURAL SCIENCES

## PhD in Food Science (35th cycle)

**Functional and biotechnological use of lactobacilli as producers of butyric and pyroglutamic acid in milk and fermented milk**


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*To my son Raffaele,*
*who conferred upon me*
*the most prestigious title:*
*the title of "mother"*

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# THESIS OVERVIEW

L'acido butirrico e piroglutammico (pGlu) sono due piccoli composti con diversi effetti benefici sulla salute umana (**Kumar & Bachhawat, 2012; Macfarlane & Macfarlane, 2012**). In particolare, l'acido piroglutammico ha attività antimicrobica, antitumorale, mitogenica, ansiolitica e antidiabetica. L'acido butirrico è, invece, uno degli acidi grassi a catena corta più studiati ed è noto per le sue proprietà antinfiammatorie. Recenti studi hanno anche dimostrato che l'acido butirrico potrebbe essere utilizzato per gestire patologie gastro-intestinali legate alla malattia da Coronavirus 2019.

Commercialmente, l'acido butirrico viene prodotto per via chimica a partire dal petrolio greggio come materia prima. Per quanto riguarda l'acido piroglutammico, esso è presente sia nei formaggi stagionati che nelle bevande fermentate. Si forma durante la fermentazione ad opera di lattobacilli termofili utilizzati come starter. La produzione di acido butirrico di origine biologica, nonostante la notevole domanda per applicazioni alimentari e farmaceutiche, è ancora un processo complesso e difficilmente controllabile. Sono stati compiuti diversi sforzi per rendere economicamente fattibile la produzione di acido butirrico e pGlu, utilizzando materie prime abbondanti/poco costose abbinate a strategie di fermentazione altamente efficienti. Tuttavia, tutti i protocolli sviluppati, in particolare per l'acido butirrico, si basano essenzialmente sull'uso di specie

*Clostridium* e ciò ne impedisce l'applicazione nell'industria alimentare. Attualmente, pochissime ricerche sono focalizzate su generi non Clostridi. In tale ottica, lo scopo di questa tesi di dottorato è stato quello di esplorare la capacità di produrre acido butirrico e piroglutammico da parte di ceppi probiotici commerciali e di colture di batteri lattici (LAB) isolate da alimenti e appartenenti a diverse collezioni. L'obiettivo è stato la definizione dell'uso funzionale e biotecnologico di alcuni ceppi batterici interessanti per l'industria alimentare. La produzione è stata valutata in terreni sintetici addizionati di lipidi e/o polisaccaridi, nel latte e nel latte fermentato per comprendere le condizioni ottimali per la produzione dei due metaboliti. Per quanto riguarda l'acido butirrico, è stato riscontrato che i ceppi di *Lb.plantarum* producevano sorprendentemente acido butirrico solo quando nel terreno era presente una fonte lipidica e non oligosaccaridica. È noto che *Lb. plantarum* possiede lipasi intracellulari ed esterasi ceppo-specifiche in grado di idrolizzare esteri contenenti acidi grassi da C4 a C6, come quelli presenti nel grasso del latte. La fibra potrebbe non influenzare l'attività butirogenica dei LAB anche nello yogurt. Per quanto riguarda l'acido piroglutammico, invece, lo yogurt, il kefir e altri latti fermentati probiotici ne contenevano da 51,65 a 277,37 mg/100 g s.s. in funzione dei batteri lattici termofili utilizzati come colture starter. Nel processo di produzione dello yogurt, l'acido piroglutammico era costante durante la fermentazione, mentre aumentava durante la fase di

stoccaggio. Inoltre, la produzione di acido piroglutammico aumentava quando la glutammina, utilizzata come precursore dai LAB, è stata aggiunta al substrato.

I risultati scientifici del presente progetto hanno portato a una comprensione più profonda della versatilità metabolica dei LAB.

Butyric and pyroglutamic acid (pGlu) are two small compounds with several beneficial effects on human health (**Kumar & Bachhawat, 2012; Macfarlane & Macfarlane, 2012**). In particular, pyroglutamic acid has antimicrobial, antitumoral, mitogenic, anxiolytic and anti-diabetic activity. Butyric acid is, instead, one of the most studied short-chain fatty acids and is known for its anti-inflammatory properties. Recent studies have also shown that butyric acid could be used to manage critical illnesses related to Coronavirus disease 2019.

Commercially, butyric acid is produced using a chemical route starting from crude oil as the raw material. As far as pyroglutamic acid is concerned, it is present both in ripened cheeses and in fermented beverages. It is formed during fermentation carried out by thermophilic lactobacilli used as starters. The production of butyric acid from a biological origin, in spite of the significant demand for food and pharmaceutical applications, is still a complex process hard to control. Several efforts have been made

to make butyric acid and pGlu production economically feasible, by using abundant/inexpensive raw materials coupled with highly efficient fermentation strategies. Nevertheless, all developed protocols are, especially for butyric acid, essentially based on the use of *Clostridium* species and this prevents the application in the food industry. Currently, very few searches are focused on non-Clostridial genera.

In such light, the aim of this PhD thesis was to explore the ability to produce butyric and pyroglutamic acid by commercial probiotic strains as well as of lactic acid bacteria (LAB) cultures isolated from foodstuffs and belonging to different cultures collections. The goal was the definition of functional and biotechnological use of some bacterial strains interesting for the food industry. Production has been evaluated in synthetic media with lipids- and/or polysaccharides-added, in milk and fermented milk to understand the optimal conditions for metabolites production. As for butyric acid, it was found that *Lb.plantarum* strains surprisingly produced butyric acid only when there was a lipid and non-oligosaccharide source in the medium. It is known that *Lb. plantarum* possesses strain-specific intracellular lipases and esterases able to hydrolyse esters containing C4 to C6 fatty acids, such as those occurring in the milk fat. Fiber may not affect the butyrogenic activity of LAB also in yoghurt. As regards pyroglutamic acid, however, yoghurt, kefir and other probiotic fermented milk contained it ranging from 51.65 to 277.37mg/100g d.m. as a function of the

thermophilic lactic acid bacteria used as starter cultures. In the yoghurt production process, pyroglutamic acid was constant during the fermentation phase and increased during the storage phase. Moreover, the production of pyroglutamic acid increased when glutamine, used as a precursor, was added to the substrate.

The scientific results of the present project led to a deeper understanding of the metabolic versatility of LABs.

**References**

Kumar, A., & Bachhawat, A. K. (2012). Pyroglutamic acid: throwing light on a lightly studied metabolite. *Current Science*, 288-297.

Macfarlane, G. T., & Macfarlane, S. (2012). Bacteria, Colonic Fermentation, and Gastrointestinal Health. *Journal of AOAC International*, 95(1), 50–60.

# CHAPTER 1. GENERAL INTRODUCTION

## 1.1 Butyric acid

Butyric acid, also called butanoic acid according to IUPAC terminology, is a carboxylic acid composed of 4 carbon atoms (**Figure 1.1**). It belongs to the class of SCFA (short-chain saturated fatty acids) together with acetic acid and propionic acid.

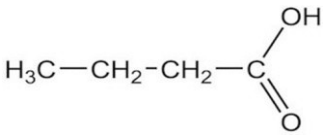


**Figure 1.1** Chemical structure of butyric acid.

In purified form, it is presented as a colourless oily liquid, soluble in water, ethanol and ethyl ether, with a melting point of -7.9 ° C and boiling point of 163.5 ° C.

It is found as an ester of glycerol in animal fats (in butter the concentration of tributyrin constitutes 3-4% of its mass) and in vegetable oils. It is characterized by an acrid taste and an unpleasant odour if present in high concentrations. In low concentrations, it is responsible, together with its esters, for the characteristic aroma of some cheeses. In free form, it is found following the fat hydrolytic rancidity processes or is formed in the fermentation processes of sugars operated by anaerobic microorganisms (butyric fermentation).

It is one of the components of sweat (it is formed because of the bacterial degradation of triglycerides present in the skin and is partially responsible for the characteristic odour); it is also found in the muscles. It is also synthesized on an industrial scale as it is widely used in various sectors: it is used to produce plastics, plasticizers, surfactants, textile auxiliaries and biofuels; its esters are used as additives in foods, perfumes, paints, pharmaceuticals and disinfectants.

### 1.1.1 Effects of butyric acid on human health

Butyric acid serves primarily as an energy source for colonic epithelial cells and, due to its powerful regulatory effects on gene expression, gives rise to a wide range of biological effects (**Table 1.1**). Numerous studies conducted in recent years have confirmed the different mechanisms of action of butyric acid and identified new target tissues (**Hamer *et al.*, 2008**).

**Table 1.1** Physiological effects of butyric acid produced by bacteria in the colon (**Macfarlane & Macfarlane, 2012**)

| Action field | Physiological effect |
|---|---|
| Immune system | • Inhibits the inflammatory response;<br>• interacts directly with the immune system;<br>• increases the production of monocytes;<br>• induces apoptosis of inactive neutrophils |
| Carcinogenesis | • Induces apoptosis of colon cancer cells; |

| | |
|---|---|
| | • inhibits the progression and migration of cancer cells;<br>• modulates genes associated with colon epithelial cell proliferation, differentiation and apoptosis |
| Oxidative stress | • Protects against the harmful oxidative effects of $H_2O_2$;<br>• increases the production of antioxidants such as glutathione;<br>• modulates oxidative stress genes in colon cells; |
| Intestinal mucosa | • Implements the barrier effect of the intestinal mucosa and decreases the permeability of the colon epithelium;<br>• reduces the translocation of *E. coli* into colon epithelial cells |
| Colon | • It increases the expression of peptides involved in the regulation of appetite, favoring the sense of satiety;<br>• regulates enteric neurons and controls intestinal motility;<br>• stimulates the absorption of $H_2O$ and electrolytes |

Butyric acid strengthens the barrier effect of the intestinal mucosa by stimulating the production of mucin (**Willemsen *et al.,* 2003**). It has been proven to increase peristaltic efficiency by improving the contractility of smooth muscle tissue of the colon and regulating intestinal neurotransmission, especially in the case of slowed peristalsis (**Bajka *et***

***al.*, 2010)**. Furthermore, all SCFAs limit the active secretion of water, sodium chloride and ions from intestinal epithelial cells (**Knudsen *et al*., 2003**). These mechanisms of action appear to be useful in the treatment of intestinal disorders, including constipation and diarrhoea.

Butyric acid has proven anti-inflammatory effects: it inhibits the production of pro-inflammatory cytokines by macrophages and monocytes (**Zhang *et al*., 2009**).

Butyric acid has been hypothesized to reinforce the barrier effect of the colon by increasing the production of mucin and antimicrobial peptides. It has also been shown to reduce the permeability of the intestinal epithelium by strengthening the intestinal junctions (tight-junction).

The anticancer effect of butyric acid is due to its ability to induce apoptosis in colon cancer cells. Apoptosis versus necrosis is a response to mild cellular injury. It is also a mechanism by which excess cells are removed during development to keep tissue size limited. Developmental apoptosis is often an indicator of programmed cell death. Since it is one of the main mechanisms by which organisms remove inadequate, redundant or damaged cells, it can be considered an innate cellular defence against carcinogenesis. Thus, many apoptosis initiators, such as butyric acid, are considered excellent therapeutic agents (**Smith *et al.*, 2009**).

The ability to induce apoptosis is probably linked to its influence on gene expression: butyric acid has been associated with the inhibition of the functions of the enzyme histone deacetylase (HDAC); consequently, it promotes an acetylated state of histones in the cell. Due to the neutralization of electrostatic charge interactions, acetylated histones have a lower affinity for DNA than non-acetylated histones. It is generally thought that transcription factors (tumour suppressors) cannot access regions where histones are closely associated with DNA (such as heterochromatin, which is not acetylated). Thus, butyric acid is thought to increase transcriptional activity towards those factors typically silenced or inhibited due to deacetylase activity.

#### 1.1.1.2 Butyric acid and Covid-19

The covid-19 disease is a new health crisis that is threatening humanity. Although it originated in the Chinese province of Hubei in late 2019, it has now spread to many countries around the world (**Wang *et al*., 2020**).

The pandemic is caused by the new betacoronavirus, now called SARS-Cov-2. (**Lake, 2020**).

Common symptoms include fever, dry cough, and difficulty breathing in most cases and headache, muscle aches, diarrhoea, loss of smell and taste to a lesser extent (**Xydakis *et al.*, 2020**).

Answers could come from the microbiota for a new credible therapeutic option or, at least, an adjuvant therapeutic choice to mitigate, based on robust scientific evidence, the disease. It is now well known that the so-called Spike protein, present on the outer surface of Sars-CoV-2, is the weapon with which the latter attacks the cell. But to enter the cell, the virus must find a way of entry which, in this case, is provided by a specific receptor represented by the Ace-2 protein, present in the host's cells. Once the virus has entered the cell, it also seems to make use of the support of some human metabolic pathways or a chain of chemical reactions activated and modulated, specifically, by the enzyme histone deacetylase (HDAC). It follows that blocking this enzyme can represent an interesting therapeutic approach targeted precisely against Sars-CoV-2 infection. Proof of this is the proliferation of experimental studies already started and aimed at identifying anti-Covid-19 drugs aimed at acting precisely on the metabolic pathway modulated by HDAC. Among the short-chain saturated fatty acids produced by the intestinal microbiota, butyric acid appears to have, among other effects, that of inhibiting the HDAC enzyme; blocking it, can interfere with the coupling and, therefore, with the penetration of Sar-CoV-2 into the human cell.

### 1.1.2 Production of butyric acid

The bacterial species that produce butyrate are *Clostridium* spp., *Eubacterium* spp., *Fusobacterium* spp., *Butyrivibrio* spp., *Megasphaera elsdenii, Mitsuokella multiacida, Roseburia intestinalis, Faecalibacterium prausnitzii* and *Eubacterium hallii.*

The formation of butyrate starts from the condensation of two molecules of acetyl-CoA and the consequent reduction to butyryl-CoA. Bacteria that use lactate can produce acetyl-CoA from the latter (**Duncan *et al.,* 2004).** In this so-called "classic" pathway, the enzymes phosphotransbutyrilase and butyrate kinase then convert the butyryl-CoA into butyrate (**Louis & Flint, 2009**).

However, another pathway has recently been discovered in which butyryl-CoA is converted to butyrate by butyryl-CoA: acetate-CoA-transferase. It is used for the conversion of exogenous acetate. This finding is supported by studies demonstrating a cross-feeding action between acetate-producing bacteria (eg *bifidobacteria*) and butyrate producers (**Duncan *et al*., 2002**).

This alternative pathway seems to dominate over the classic one: the latter is used by the major butyrate-producing species such as *Roseburia, Eubacterium rectale, Eubacterium hallii* and *Faecalibacterium prausnitzii* (**Venema, 2010**).

Butyric acid mainly derives from the decomposition of pentoses contained in wholemeal products (wheat bran, wholemeal bread, pasta and brown rice), legumes, vegetables and fruit. In particular, resistant starch is considered butyrogenic and examples of products rich in this dietary fiber are partially ground cereals and seeds, uncooked and cooked and chilled potatoes, green bananas, vegetables and legumes.

Recent studies carried out on animals and humans suffering from colon cancer have shown a correlation between a diet supplemented with wheat bran and a reduction in the proliferation of cancer cells. **Boffa *et al.* (1978)**, measured a corresponding increase in butyric acid levels in the colon. The data suggest that an increase in daily fiber intake and subsequent production of butyric acid would be useful as a measure against colon cancer, thanks to the ability of butyric acid to induce apoptosis in colon cancer cells (resulting in their removal).

Furthermore, *in vitro* experiments have shown that the lactate produced by lactic bacteria is used by some narrow anaerobic butyrate-producing bacteria belonging to cluster XIV of *Clostridia,* for the production of high concentrations of butyric acid (**Louis & Flint, 2009**). This cross-feeding mechanism could explain why the administration of lactic acid bacteria in patients with inflammatory bowel diseases can, in some cases, be a solution, thanks to the *in situ* production of butyric acid.

## 1.2 Pyroglutamic acid

Pyroglutamic acid, also known as 5-oxoproline, 2-pyrrolidone 5-carboxylic acid (pGlu), or pidolic acid, consists, instead, of an amino acid derivative of glutamic acid (**Figure 1.2**); the precursor molecule cyclizes starting from the free amino function, forming a lactam (**Kumar & Bachhawat, 2012**). This organic acid was first identified and characterized in 1882 by the Austrian chemist Ludwig Camillo Haitinger (1860-1945); he observed that, by applying high temperatures equal to 180 ° C, glutamate is converted into pyroglutamic acid with the release of a water molecule (**Haitinger, 1882**).

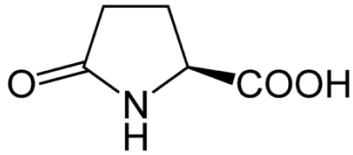


**Figure 1.2** Chemical structure of pyroglutamic acid.

Subsequently, it was shown that the cyclization of the amino acid L-glutamine in pGlu follows pseudo-first-order chemical kinetics and is positively influenced by the acid and sub-acid pH, in a range of 3 to 6, typical of many foods (**Terry & Chang, 1975**). Some studies have shown that pyroglutamic acid is relatively stable at high temperatures, while an increase in the pH of the medium can alter the antimicrobial activity of the molecule towards some bacterial species (**Yang *et al.,* 1996**). Pidolic acid is a ubiquitous metabolite in nature where, due to the presence of the chiral

carbon atom in position 2 carrying the carboxylic function, it can exist in two distinct enantiomeric forms: D-glutamic acid and L-pyroglutamic acid, with respective stereogenic center configuration 2R or right-handed (+) and 2S or left-handed (-), respectively. Both molecules, pyroglutamic acid and glutamic acid, are chiral in nature; the carbon atom involved is highlighted with an asterisk in **figure 1.3**.

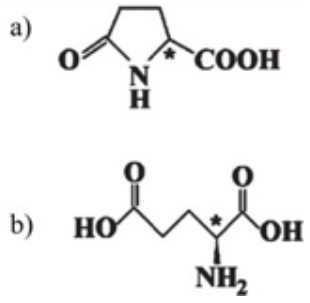


**Figure 1.3** Pyroglutamic acid (a) and glutamic acid (b)

Given the instability of glutamate and glutamine in an aqueous environment, for a long time, it was believed that the synthesis of pyroglutamic acid in tissues of animal and plant origin was attributed solely to the spontaneous and non-enzymatic genesis of the molecule (**Kumar & Bachhawat, 2012**). Only starting from the first half of the twentieth century have further possible synthetic pathways, of an enzymatic nature, been detected for the metabolite; one of these involves the genesis of pyroglutamic acid (5-oxoproline) starting from glutathione (**Connel & Hanes, 1956**), in fact, pGlu is a reaction intermediate of the glutathione synthesis and degradation cycle (GSH), also defined " γ-glutamyl cycle ".

As shown in **Figure 1.4**, the degradation of GSH into γ-glutamyl-amino acids occurs first by the γ-glutamyl-transpeptidase enzyme.

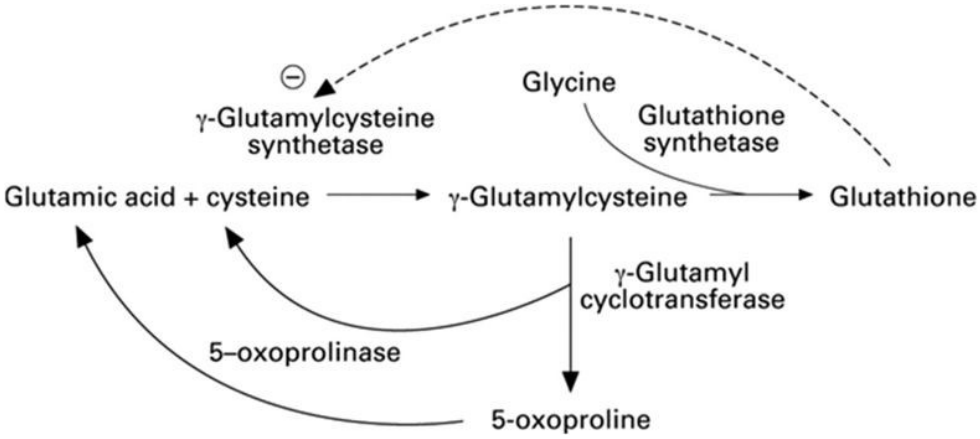


**Figure 1.4** Γ-glutamyl cycle and involved enzymes (**Abkur *et al.*, 2014**).

In 1970, it was the American biochemist Alton Meister who integrated the synthesis of pyroglutamic acid and its degradation as part of the GSH cycle **(Meister, 1973**). The cyclization of glutamate, as well as spontaneously, can also occur in the presence of enzymes such as glutamate 5-kinase, γ-glutamylcysteine-synthetase or glutamine synthetase (**Figure 1.5**). In all three cases, the molecule is activated in phosphorylated glutamate in the presence of an electron acceptor, which consists respectively of cysteine, ammonia and NADPH - nicotinamide adenine dinucleotide phosphate (**Krishnaswamy *et al.*, 1960**). The phosphorylated glutamate produced, being a highly unstable reaction intermediate, spontaneously tends to cyclize into pyroglutamic acid.

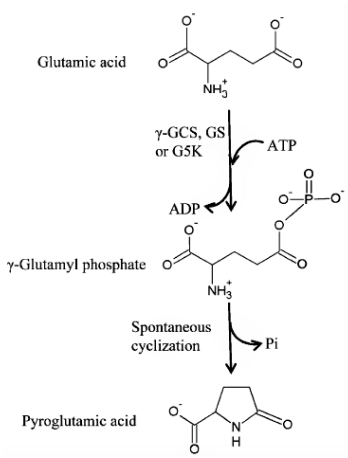


**Figure 1.5** Scheme of the synthesis of 5-oxoproline (pGlu) from the partial reaction of the enzymes γ-GCS (γ-glutamylcysteine synthetase), GS (glutamine synthetase) and GSK (glutamate 5-kinase) (**Kumar & Bachhawat, 2012**)

Greater clarity on the nature and *in vivo* synthesis of pidolic acid was obtained only after thorough biochemical investigations of the enzymes involved. Γ-glutamyl-cyclotransferase was purified in 1969 and, after a few years, 5-oxoprolinase; the primary protein structure of these enzymes has only recently been described. The lactam cyclization reaction of L-glutamate into L-pGlu by γ-glutamyl-cyclotransferase (γ-GCT) probably represents the main biosynthetic pathway of the molecule in mammals (**Orlowski & Meister, 1973**). As regards the 5-oxoprolinase enzyme, the latter has been detected and isolated in a broad spectrum of living organisms such as bacteria, plant and animal species (in particular mammals); the latter catalyzes the hydrolysis of pyroglutamic acid into

glutamic acid which, being available again, can re-enter the γ-glutamyl cycle or perform specific cellular functions.

In eukaryotes, this ATP-dependent dimeric enzyme is approximately 280 kDa in size. The relative mechanism of conversion of pGlu into glutamate first involves the phosphorylation of pyroglutamic acid in phosphorylated 5-oxoproline (by hydrolysis of ATP), then hydrolyzed into γ-glutamyl phosphate; this reaction intermediate will in turn undergo hydrolysis into glutamate and inorganic phosphate **(Seddon and Meister, 1986).**

The gene encoding 5-oxoprolinase was identified and sequenced in 1996 in mammalian cells; later, a new type of 5-oxoprolinase was also isolated from prokaryotic organisms, specifically from the gram - *Alcaligenes faecalis* bacterium. The enzyme has unusual characteristics compared to the previous ones observed, including a relatively small size (46 kDa) and ATP-independent activity; homologous enzyme forms were found only in a small number of bacterial groups (**Nishimura *et al.*, 2000**). Recently, the purification of the enzyme from *Saccharomyces cerevisiae* has allowed to obtain a more detailed analysis in terms of biological structure and function (**Kumar & Bachhawat., 2010**).

Pidolic acid in nature can be commonly found in free form but also as an amino residue of many biologically active proteins and peptides, where it

is not formed directly from the transcription and translation of DNA but only following the cyclization of glutamine or N- glutamate terminal; this type of reaction can occur spontaneously or in the presence of the glutamine-cyclase enzyme, where cyclization is favored by the glutamine substrate. Many structural proteins of animal origin, such as collagen, fibrinogen and fibrin, exhibit a similar terminal amino function; in this case, the pyroglutamic acid preserves its protein structure, counteracting its degradation (**Fietzek *et al.*, 1974**).

Therefore, among the different mechanisms of generation of pyroglutamic acid in the cellular environment (**figure 1.6**), the release of the molecule from the enzymatic lysis of specific proteins or peptides must also be considered.

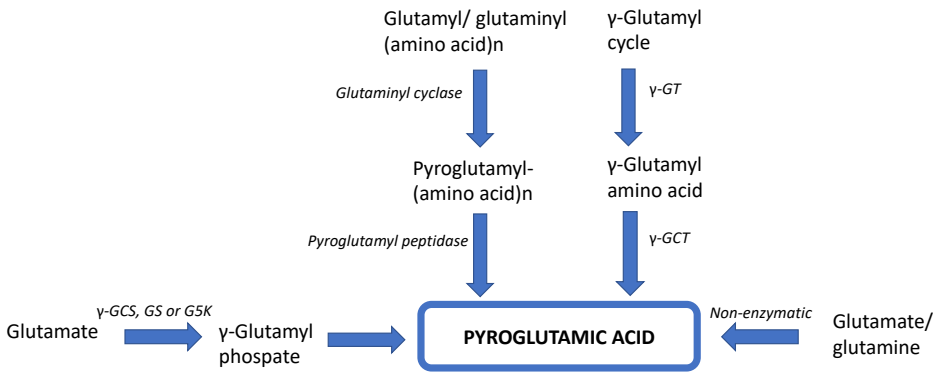


**Figure 1.6** Schematic representation, adapted from Kumar & Bachhawat (2012), of different routes of pGlu generation. G5K, Glutamate 5-kinase; γ-GT, γ-Glutamyl transpeptidase; γ-GCT, γ-Glutamyl cycoltransferase; γ-GCS, γ-Glutamylcysteine synthetase; GS, Glutamine synthetase.

PGlu removal is mediated by PCP exopeptidase (Pyrrolidone Carboxylic Peptidase), also known as PYRase or Pyroglutamyl peptidase. The enzyme has been detected in human tissues but also in numerous bacterial and plant species; the biochemical investigation of its properties allowed to distinguish and characterize two classes, which are represented by type I PYRase (in bacteria and animals) and type II PYRase, exclusive to the animal kingdom and isolated from serum. The first application of PYRase was the sequencing of some proteins and peptides but only the subsequent development of specific chromogenic substrates for the enzyme allowed its use in bacterial identification (**Awade *et al.*, 1994).**

Generally, aminopeptidases are exopeptidases that selectively release N-terminal amino acids from polypeptides and proteins; in bacteria, these particular enzymes are localized at the level of the cytoplasm and membrane, where exopeptidase activity is associated with cell development, or are secreted in the extracellular environment (**Gonzales & Robert-Baudouy, 1996**).

Briefly summarizing the set of possible metabolic pathways through which the generation of pyroglutamic acid occurs, it is possible to distinguish:

1. Non-enzymatic hydrolysis of glutamate;

2. Enzymatic activation of glutamic acid / glutamate in γ-glutamyl phosphate and consequent spontaneous cyclization of the unstable intermediate in pGlu (incomplete reaction following activation of GSH);

3. Degradation of GSH into γ-glutamyl amino acids, a substrate for the γ-GCT enzyme (γ-glutamyl cycle);

4. Release from the N-terminus of peptides and proteins mediated by exopeptidases.

### 1.2.1 Physiological role of pGlu

Pyroglutamic acid as a free cellular metabolite is abundant in human tissues such as the epidermis (**Marstein *et al.,* 1973**), plasma, eyeball, cerebral cortex (**Abraham *et al.,* 1981; Van Der Werf & Meister, 1975**) and cerebrospinal fluid in a relatively high concentration of $10^{-5}$-$10^{-3}$ M; although enzymes capable of forming pyroglutamic acid are present in many organs, the epidermis seems to be the only tissue in which the substance accumulates (**Marstein *et al.,* 1973).** When pGlu is ingested through certain foods or the endogenous levels of the metabolite become elevated, pyroglutamic acid is naturally excreted in the urine in amounts ranging from 0.5 mg to 5 mg per day. except subjects suffering from 5-oxoprolinuria (inherited or caused by pathological conditions) in which a

deficiency of the enzymes 5-oxoprolinase and GSH-synthetase lead to an increase in daily secretion up to 50 mg (**Ristoff & Larsson, 2007**). There are numerous human proteins and peptides bearing a pGlu residue (pyroglutamic acid), many of which perform important physiological functions (**table 1.2**); in fact, about half of the antibodies reported in the literature expose a glutamic acid to the terminal amino function of the peptide chain.

Cyclization of the molecule into the cyclic lactam pyroglutamic acid, making the γ-immunoglobulin antibody resistant to aminopeptidases, would lead to an increase in its half-life *in vivo*, even if this has not currently been demonstrated (**Chelius *et al.*, 2006**). The impact of pGlu on antibody activity needs further clarification, especially to compensate for the microheterogeneity of antibodies and proteins synthesized for therapeutic purposes by the biotechnology industry, on which loss of control over the synthesis process may depend (**Liu *et al.*, 2011**).

Furthermore, pGlu plays a decisive role in the functionality of many neuropeptides, including thyrotropin (TRH) and luteinizing hormone (LHRH) releasing hormones; in fact, structural alterations of the organic acid in these molecules or substitutions to the lactam ring cause a significant decrease in both receptor activity and affinity. In particular, it is known that the binding of the TRH hormone to the receptor is mediated by

the interaction between the specific sites and the carbonyl ring exposed by the pGlu residue (**Perlman *et al.*, 1994**).

In addition to preserving the structure of peptides and proteins from degradation, N-terminal pyroglutamic acid increases its hydrophobicity and at the same time causes a decrease in the solubility of the molecule. Both effects of the metabolite are involved in the deposition of β-amyloid plaques (amyloidogenic peptide Aβ), of which pGlu itself is the main constituent; as widely known, Aβ plaques are closely related to the genesis of neurodegenerative diseases such as Alzheimer's (**Wirths *et al.*, 2009**). Numerous studies have shown how the presence of pyroglutamic acid in these proteins can cause a propensity for aggregation between fibrils and has a direct role in the pathogenesis, in fact glutamine-cyclase, an enzyme involved in the conversion of glutamine into pyroglutamic acid lactam, undergoes up-regulation in the cerebral cortex of Alzheimer's patients; *in vivo* studies confirm that inhibition of the enzyme, in an experimental model conducted on rats, induces a decrease in the level of pGlu-modified peptides and attenuates the effects related to neurodegeneration (**Schilling *et al.*, 2008**).

The presumed neurotoxicity of pidolic acid has been suggested by numerous studies describing its involvement in Huntington's disease; the relationship between this pathology and the increase in plasma pGlu levels

is however unusual, due to the simultaneous intrastriatal depletion of L-pyroglutamic acid (**Uhlhaas & Lange, 1988**).

The physiological function of pyroglutamic acid in free form is still unclear; being an analogue and potential precursor of glutamate, pGlu is involved in all processes involving the presence of the amino acid, especially in the brain (**Moret &Briley, 1988**). Pyroglutamic acid is, in fact, able to bind to glutamate receptors, inhibiting its uptake to the synaptic neuron, and causing a consistent loss of brain cells in the adult rat (**Dusticier *et al.,* 1985**). The ability of pidolic acid to cross the blood-brain barrier via a Na + dependent monocarboxylate transport system (**Miyauchiet *et al.,* 2010**), explains its considerable accumulation both in the blood and in the brain after oral administration (**Caccia *et al.,* 1982**).

Despite the evidence drawn from the studies conducted, the neuronal effects of pGlu as a free acid prove controversial; it has been shown that the molecule can prevent scopolamine-induced amnesia and counteract the decline in learning capacity linked to aging. The administration of pyroglutamic acid as a therapeutic agent stimulates the cholinergic mechanism linked to memory and learning, inhibiting the memory loss induced by alcohol consumption in rats (**Drago *et al.,* 1988**). The positive effects of the molecule on learning were also observed in both juvenile and adult rats (**Drago *et al.,* 1987; Drago *et al.,* 1988**). From the results

obtained from clinical trials, conducted on humans, pGlu induces an improvement in cognitive functions both in cases of alcoholic encephalopathy (induced by chronic alcohol consumption) in the form of arginine salt (**Sinforiani *et al.*, 1985**) and in Alzheimer's patients (**Grioli *et al.*, 1990**).

The recommended daily dose of a preparation of L-pyroglutamic acid in the form of arginine salt is between 400 and 1000 mg; the applications of the latter concern cases of senility, memory difficulties, brain diseases related to alcohol abuse and dyslexia (**Pfeiffer & König, 2009**).

**Table 1.2** Main peptides and proteins of animal-human origin bearing pGlu as the N- residueterminal (Kumar & Bachhawat, 2012).

| **Protein / Peptide** | **Amino acid sequence** |
|---|---|
| TRH | pGlu-His-Pro-NH2 |
| TRH-like peptides (prostate) | pGlu-Glu-Pro-NH2 |
| Anorectic peptide | pGlu-His-Gly-OH |
| Eisenina | pGlu-Glu-Ala-OH |
| Mitosis inhibitor peptide (colon) | pGlu-Glu-His-Gly-OH |
| Mitosis inhibitor peptide (epidermis) | pGlu-Glu-Asp-Cys-Lys-OH |
| Vasoactive polypeptide | pGlu-Val-Pro-Gln-Trp |
| LHRH | pGlu-His-Trp-Ser-Tyr-Gly-Leu-Gln-Pro-Gly-NH2 |
| GnRH-II | pGlu-His-Trp-Ser-Hys-Gly-Trp-Tyr-Pro-Gly-NH2 |
| Eleidosine | pGlu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH2 |
| Neurotensin | pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-NH2 |

| | |
|---|---|
| Fibrinopeptide B | pGlu-Gly-Val-Asn-Asp-Asn-Glu-Glu-Gly-Phe-Phe-Ser-Ala-Arg |
| Gastrin | pGlu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met- |
| B-amyloid peptide Aβ11 (pE) -40/42 | pGlu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser- |
| Peptide β-amyloid Aβ3 (pE) - 40/42 | pGlu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val- |
| Orexin A | pGlu-Pro-Leu-Pro-Asp-Cys-Cys-Arg-Gln-Lys-Thr-Cys-Ser-Cys-Arg-Leu- |
| Apelina | pGlu-Arg-Pro-Arg-Leu-Ser-His-Lys-Gly-Pro-Met-Pro-Phe |

In recent years, particular attention has been paid to the clinical potential of human placenta extract, administered in Japan for over 50 years to improve the liver function of patients with hepatitis or cirrhosis; the stimulating effect on liver regeneration has been demonstrated *in vivo* and *in vitro*, both for placental extracts obtained by acid hydrolysis (by hydrochloric acid - HCl) and by enzymatic digestion (**Liu *et al.,* 1995**) but only recently it has been possible to purify and identify the active ingredient responsible for this effect: pyroglutamic acid (**Inoue *et al.,* 2015**). *In vitro* studies conducted on primary rat hepatocytes (isolated from the liver of male subjects aged six to seven weeks) have allowed us to investigate the presence of pathways through which pyroglutamic acid induces DNA synthesis; this effect was monitored using the ELISA technique (enzyme-

linked immunosorbent assay) in hepatocytes incubated for 22 hours with pGlu at a concentration of 10 mM.

The mitogenic effect of pGlu is dose-dependent while the mechanism of action is mediated by the MAPK (mitogen-activated protein kinase) signaling pathway; this was indirectly demonstrated by the inhibitory effect of PD98059 (2′-amino-3′-methoxyflavone), a flavone derivative capable of specifically inhibiting type 1 MAPKs activated by pyroglutamic acid (**Inoue *et al.,* 2015**). Furthermore, pyroglutamic acid induces an increase in phosphorylation of extracellular signal-regulated type 1 and type 2 kinases (ERK1 / 2), thus involving signaling pathways having a critical role in the development of human hepatocellular carcinoma. (**Liu *et al.,* 2006**). The results obtained from this study suggest future applications of pyroglutamic acid as a pharmacological agent that stimulates the multiplication of hepatocytes (**Inoue *et al.,* 2015**).

Although the preventive effects of pGlu towards the genesis of tumours and metastases are known (**Kimura *et al.,* 2004**), the molecule is not able to modify the synthesis of DNA in carcinoma cells and cells of the endothelium of the umbilical vein; the mitogenic effect could be specific for each cell type, therefore other studies are needed to define this selectivity (**Inoue *et al.,* 2015**).

### 1.2.2 Effects on human health of pidolic acid

There are numerous pharmacological applications of pyroglutamic acid currently known; the esters of this organic acid have properties such as to increase the absorption of active principles with poor dermal permeability (**Jose &Takeru, 1991**) or stimulating hair growth (**Gibson & Scott, 1992**). In the field of chemical synthesis, pGlu is widely used both for selective enantiomeric synthesis (**Petersen *et al.,* 1984**) and as a versatile biosynthetic building block for alkaloids, pharmacologically active ingredients, and other natural products (**Nájera & Yus, 1999**).

In neurology, the stimulating effect of the molecule was discovered in 1984; intravenous (3.2 - 6.4 mg) and intraperitoneal (1g /kg) administration of pyroglutamic acid is, in fact, capable of inducing mild sedation, modifying the electrocorticogram - EEG and increasing the release of neurotransmitters such as acetylcholine and γ-aminobutyric acid (GABA) from the cerebral cortex of guinea pigs (**Antonelli *et al*., 1984**). Pidolic acid, as a key metabolite of neurotransmission, crosses the blood-brain barrier and, having reached the central nervous system - CNS, is intercepted by terminal glutamatergic neurons; this mechanism explains the antagonistic activity of pyroglutamic acid towards the glutamic acid precursor, competing for the same receptor sites and thus altering their absorption (**Dusticier *et al*., 1985**). At the doses indicated above (**Antonelli**

***et al*., 1984**), pyroglutamic acid stimulates GABAergic transmission but this effect is stereospecific, in fact, while L-pGlu is able to induce specific effects at the level of the cerebral cortex, the dextrorotatory form D-pGlu is inactive (**Beni *et al*., 1988**); molecules with similar activity are usually associated with anxiolytic properties (**Haefely *et al*., 1981**).

Studies conducted on experimental animal models have shown that, although no enantiomeric form of pyroglutamic acid modifies the locomotor activity of the rat, L-pGlu (administered at doses of 500 mg / kg and 1000 mg /kg) exhibits an anxiolytic effect without modify the levels of 5-HT serotonin (5-hydroxytryptamine) and 5-hydroxyindolacetic acid (5-HIAA) in the rat cortex and hippocampus; furthermore, the activity of L-pyroglutamic acid is not affected by the simultaneous administration of a benzodiazepine antagonist ("Ro 15-1788", also known as "Flumazenil"), suggesting different mechanisms of action from this class of drugs and by agonists of the 5-HT1a serotonergic receptor (**Beni *et al*., 1988**).

The inhibitory effects of pyroglutamic acid on the proliferation of metastatic tumor cells were investigated *in vivo* and *in vitro*, on Lewis lung carcinoma cells isolated from rats, by assaying isolates of sodium pyroglutamate and ergosterol obtained from the fruiting body of the fungus *Agaricus blazei* Murrill.; the species belongs to the Agaricaceae family and has an antitumor, antidiabetic and counteracting action against chronic

hepatitis and hyperlipidemia, mainly associated with β-D-glucans and glycoproteins. Its use for preventive purposes (in the form of an extract in hot water) is widespread in Brazil and Japan, for which the annual production of the dried product amounts to 400 tons.

The presence of the metabolite has also been found in other species belonging to the order of *Basidiomycetes,* such as: *Lentinus edodes, Flammulina velutipes, Lyophyllum aggregatum, Pleurotus ostreatus, Phellinus linteus, Ganoderma lucidum, Hericium erinaceus, Coriolus versicolor, Agaricus campestrosa, Sparassis erispa, Schizophyllum commune, Tremella fuciformis* Berkeley and *Cordyceps sinensis* (**Kouge *et al.*, 2011**)

The antidiabetic activity manifested by pGlu was tested *in vivo* through an experimental animal model that included rats with unaltered glucose metabolism (Wistar rats), diabetic rats (Goto-Kakizaki) and both obese and diabetic rats (KK-A y); the rats of the three types were separately subjected, for a defined time, to a basal diet (forming the control group) and to a diet that includes 0.05% pyroglutamic acid. For non-diabetic rats, it was shown that the amino acid does not bring about changes in the lipid level or induce liver inflammation while, in diabetic subjects, the epididymal adipose tissue and the serum levels of glucose, insulin, LDL cholesterol (low density lipoprotein) and triglycerides were significantly reduced compared

to the control group; inhibition of lipid accumulation mediated by the intake of pyroglutamic acid occurred only in animals not suffering from severe obesity (**Yoshinari & Igarashi, 2011**).

These metabolic effects have been associated with the regulation that pGlu induces in the gene expression of some molecules; in fact, there is an increase in glucose tolerance due to the suppression of the gene that codes for the enzyme glucose-6-phosphatase (G6Pase), while the reduction in the plasma level of triglycerides is mediated by the negative effect that the pidolic acid exerts on the gene expression of angiopoietin-like-4, a protein that modulates the homeostasis of triacylglycerols. Negative regulation of expression for the enzyme glucose-6-phosphatase (G6Pase), reducing plasma glucose levels. Given the close correlation between tumor necrosis factor α (TNF-α) and insulin resistance that occurs in cases of obesity (**Hotamisligil *et al.*, 1993**), the effect of pGlu on the level of this factor was investigated; the results obtained showed a decrease in the level of TNF-α, probably due to the synthesis of the factor by macrophages migrating to hypertrophic fat cells (**Suganami *et al.*, 2005**). Ultimately, a diet that involves the intake of pyroglutamic acid can mitigate diabetes through the decrease of insulin resistance, liver and plasma lipid levels and the regulation of both glucose expression and lipid metabolism (**Yoshinari & Igarashi, 2011**).

As regards the damage to the optic nerve, pyroglutamic acid has been reported to stimulate Na + -dependent amino acid transport, including excitatory amino acid transporters 1-3 (EAAT1-3) at the blood-brain barrier albuminoid membrane. From a physiological point of view, these transporters are not only fundamental for the homeostasis and transport of glutamate from the extracellular fluid to presynaptic cells (**Hawkins *et al.,* 2006**) but they play a critical role; in fact, the inhibition of EATTs is directly related to the death of retinal ganglion cells in apoptosis (**Vorwerk *et al.,* 2000**).

Moreover, from previous studies it has been shown that pyroglutamic acid exhibits antimicrobial activity comparable to other organic acids produced during fermentation, first of all lactic acid; it has been shown that the inhibitory effect of pGlu towards bacterial cells and spores is of the substrate-dependent type, in particular, towards some species (belonging to the genus *Pseudomonas, Enterobacter, Bacillus* and *Clostridium*), it is higher in culture broth rather than in solid medium. For gram-negative bacteria, a higher sensitivity to pyroglutamic acid was found than for gram-positive bacteria while all lactic bacteria show resistance to the molecule; in fact, the growth of some strains of the genus *Pseudomonas* and *Enterobacter* is inhibited at pGlu concentrations below (**Yang *et al.,* 1997**).

Some strains of the *Saccharomyces cerevisiae* yeast are able to metabolize the pyroglutamic acid present in the growth substrate, reducing its concentration by 85% in aerobic conditions and by 30% in anaerobiosis; mixed yeast cultures made up of species belonging to the genus *Saccharomyces, Schizosaccharomyces, Zygosaccharomyces, Klyveromyces, Debaryomyces, Cryptococcus* and *Torulaspora* are, instead, able to degrade pGlu by 90% in the presence of $O_2$, with a faster rate than that shown by cultures consisting exclusively from *S. cerevisiae*. This investigation could be useful in the development of future applications of pGlu metabolizing yeasts, in order to reduce their negative influence on the flavor profile of many foods (**Pfeiffer & König, 2009**).

### 1.2.3 Pyroglutamic acid in food

In nature, pidolic acid is a ubiquitous amino acid, the presence of which in its native state is to be found, as shown in **Table 1.3**, above all in many fruits, vegetables (**Airaudo *et al.,* 1987**), products of animal origin (meat and dairy products), grains and some fermented foods; the latter include "lao-chao", fermented rice of the Chinese tradition (**Liu *et al.,* 2002**) and soy sauce, in which the pGlu concentration can reach values of 4831 mg/kg (**Masaaki *et al*., 1992**; **Kaneko *et al.,* 1994**; **Syuhei *et al.,* 1994**). Among the various vegetable preserves, tomato juice, a processed food in which the presence of pyroglutamic acid is known, has concentrations so high as

to reach values equal to 1000 - 2000 mg/kg (**Marconi *et al*., 2007**); in addition to the tomato (*Solanum lycopersicum*) also other plant species belonging to the *Solanaceae* family, such as the potato tuber (*Solanum tuberosum*) represent a natural source of the amino acid pGlu (**Bushway *et al.,* 1984**). Pyroglutamic acid has also been detected in plant species for food and medicinal purposes; the coffee, a stimulating drink obtained by grinding the toasted seeds of small trees belonging to the genus Coffea, it can have an organic acid content equal to 90 mg/kg - or ppm - for the beans; in the case of freeze-dried instant coffee, the pGlu level is very high (9800 ppm), especially if decaffeinated, where the concentration of pyroglutamic acid rises to 10120 ppm probably due to some stages of the production process to which the raw material is subjected . From oral administration of coffee samples rich in pyroglutamate, with and without caffeine, an important partial reversal effect of scopolamine-induced amnesia was shown in albino mice (**Maeso *et al.,* 2006**).

In the context of dairy products, cheeses are particularly rich in pyroglutamic acid, for which it has been shown that the pGlu content depends on the presence of added thermophilic lactic cultures and is directly proportional to the maturing time. This evidence is confirmed by the presence of a low pGlu content for Bagos (670 mg/kg), a long-aged Italian cheese obtained solely from the fermentation of naturally occurring

microbial species; furthermore, the results shown by previous studies suggest the potential use of the pGlu content as an index to establish the ripening time of Parmigiano-Reggiano cheese (**Panari, 1985**). In the case of Grana Padano, pyroglutamic acid in free form can be detected in large quantities from 2 to 3 months of aging, up to 6000 mg/kg after 24 months (**Mucchetti *et al.*, 2000**); while, as an N-terminal amino acid, pGlu has been identified in the bitter peptide deriving from β-casein (β-CN fragment 46-67), in different varieties of cheese (**Lemieux & Simard, 1992**). From a sensory point of view, the cyclization of the terminal amino acid has been associated with the loss of the bitter taste of the peptide (**Panari, 1985**), while aqueous solutions obtained starting from pure pyroglutamic acid (1000 mg/L) have a penetrating metallic odor and taste. salty with bitter aftertaste (**Pfeiffer & König, 2009**).

For other products belonging to the category of dairy derivatives, future investigations are necessary in order to determine the concentration in pyroglutamic acid; in the case of yogurt, a recent study has made it possible to highlight the molecule among the volatile free organic acids characterizing the metabolic profile of yogurt produced from sheep and goat milk (**Murgia *et al.*, 2019**). However, for fermented milks, there are no analysis conducted that return a quantitative determination of the pGlu.

The presence of pyroglutamic acid in sucrose, a final industrial product obtained from *Beta vulgaris* (sugar beet), particularly rich in amino-compounds (**Godshall *et al.,* 1988**), can be problematic when sugar is part of the formulation of some products (milk or fruit juice base) as the molecule is chemically active in the Maillard reaction (**Wegener *et al.,* 2017**); the latter is one of the most important non-enzymatic browning reactions in food chemistry and the relative control of the resulting products is decisive for their quality (**Yeom *et al.,* 2000**). By applying heating to a solution of L-glutamine and D-glucose, cyclization of the amino acid occurs after 40 minutes; the lactam derivative L-pGlu is much more stable and does not participate directly in the amino-carbonyl reactions but the exposed lateral carboxylic chain is particularly reactive. In the presence of the monosaccharide D-glucose, applying high temperatures to the solution for 180 minutes, the L-pyroglutamic acid shifts the browning index (evaluated by absorption at 420 nm) from 0 to 1.67. The influence of L-pGlu on caramelization is due to the formation of low molecular weight colored products; the reaction underlying the genesis of these molecules does not foresee the degradation of the amino acid but is attributed to the lateral carboxyl chain, responsible for the increase in the rate of mutarotation and isomerization of D-glucose (**Wegener *et al.,* 2017**).

In terms of technological applications, among other organic acids that exhibit the same chemical behavior (acetic acid, formic acid and lactic acid), L-pyroglutamic acid appears to be a critical contaminant in sucrose production processes due to unwanted production. of compounds that cause browning; strategies are being developed to reduce the amino acid content in order to ensure the quality of the finished product, especially if destined for long-term storage (**Wegener *et al.*, 2017**).

Pyroglutamic acid has a variable impact on the aroma of the products in which it is present, in free form or as a constituent of low molecular weight peptides that give bitter or umami flavor (**Gazme *et al.*, 2019**); in acidic foods and beverages, much of the pGlu content is generated by the high temperatures applied during some process phases but a relevant factor in the genesis of the molecule is certainly the starting concentration of the naturally present glutamic acid. There are many alcoholic beverages in which it is possible to identify pGlu as a constituent of the characterizing organic acid profile; examples are wine, in which it can reach quantities of 610 mg/L (white wine) and 220 mg/L (red wine), beer (160 mg/L) and cider, in which pGlu concentrations approaching 50 mg/L have been found (**Pfeiffer & König, 2009**). The conversion of the precursor amino acid into pyroglutamic acid also seems to be positively influenced by the conditions to which the drink is subjected during alcoholic fermentation (**Pfeiffer**

**&König, 2009**), in fact in non-alcoholic beers there is a decrease in the concentration of the metabolite up to the value of 92 mg/L (**Cortacero-Ramírez *et al.*, 2005**).

**Table 1.3** Pyroglutamic acid in food

| **Food** | **mg/kg** | **Reference** |
|---|---|---|
| Tomato juice | 1834 | Marconi *et al.,* 2007 |
| Soy sauce | 3312 | Kaneko *et al.,* 1994 |
| Coffee grain | 90 | Maeso *et al.,* 2006 |
| Instant coffee | 9800 | Maeso *et al.,* 2006 |
| Decaffeinated instant coffee | 10120 | Maeso *et al.,* 2006 |
| White wine | 610 | Pfeiffer & König, 2009 |
| Red wine | 220 | Pfeiffer & König, 2009 |
| Beer | 160 | Pfeiffer & König, 2009 |
| Non – alcoholic beer | 92 | Cortacero-Ramírez *et al.,* 2005 |
| Cider | 50 | Pfeiffer & König, 2009 |
| Bagos | 670 | Mucchetti *et al.,* 2002 |
| Grana Padano cheese (24 months of repening) | 6000 | Mucchetti *et al.,* 2002 |
| Cooked hard cheese (60 – 365 days of repening) | 1000/ 5570 | Mucchetti *et al*., 2002 |

### 1.3 Aims of the thesis

Although there are various studies reporting the content of pyroglutamic acid in various food matrices. There is a lack of information regarding fermented milks such as yogurt and kefir. Furthermore, the production process of butyric acid by the lactobacilli and above all by which substrate (fiber or fat) they use as a precursor is not clear.

Therefore, with this study, we focused first of all on creating a database currently absent in the literature on how much pyroglutamic there is in the various fermented milks already available on the market. Subsequently, in making yogurt on a laboratory scale, it was studied how this content evolves during fermentation and storage. Then, we tried to clarify the mechanism of butyric acid production by inoculating some strains of lactobacilli in milk and substrates with different ingredients.

Probiotic, prebiotic and synbiotic yogurts were then made to evaluate the content of both pyroglutamic acid and butyric acid. Finally, fermentation tests were performed with lactobacilli to clarify the strain dependency of pyroglutamic production.

## References


Abraham, G. N., Podell, D. N. (1981). Pyroglutamic acid non-metabolic formation, function in proteins and peptides, and characteristic of the enzymes effecting its removal. *Mol Cell Biochem*, 38, 181 – 190.

Abkur, T. M., Mohammed, W., Ali, M., & Casserly, L. (2014). Acetaminophen- induced anion gap metabolic acidosis secondary to 5-oxoproline: A case report. *Journal of Medical Case Reports*, 8(1), 409.

Airaudo, C. B., Gayte-Sorbier, A., and Armand, P. (1987). Stability of glutamine and pyroglutamic acid under model system conditions: influence of physical and technological factors. *J. Food Sci*., 52, 1750 – 1752.

Antonelli, T., Carlà, V., Lambertini, L., Moroni, F., & Bianchi, C. (1984). Pyroglutamic acid administration modifies the electrocorticogram and increases the release of acetylcholine and gaba from the guinea-pig cerebral cortex. *Pharmacological Research Communications*, 16(2), 189–197.

Awade, A. C., Cleuziat, P., Gonzales, T. and Robert-Baudouy, J. (1994). Pyrrolidone carboxyl peptidase (Pcp): an enxyme that removes pyroglutamic acid (pGlu) form pGlu-peptides and pGlu-proteins. *Proteins Struct. Funct. Genet*., 20, 34 – 51.

Bajka, B. H., Clarke, J. M., Topping, D. L., Cobiac, L., Abeywardena, M. Y., & Patten, G. S. (2010). Butyrylated starch increases large bowel butyrate levels and lowers colonic smooth muscle contractility in rats. *Nutrition research,* 30(6), 427-434.

Beni, M., Pellegrini-Giampietro, D. E., and Moroni, F. (1988). A new endogenous anxiolytic agent: L-pyroglutamic acid. *Fundamental & Clinical Pharmacology,* 2(2), 77 – 82.

Boffa, L. C., Vidali, G., Mann, R. S., & Allfrey, V. G. (1978). Suppression of histone deacetylation in vivo and in vitro by sodium butyrate. *Journal of Biological Chemistry,* 253(10), 3364-3366.

Bushway, R. J., Bureau, J. L., and Mcgann, D. F. (1984). Determinations of Organic Acids in Potatoes by High Performance Liquid Chromatography. *Journal of Food Science*, 49(1), 76 – 77.

Caccia, S., Garattini, S., Ghezzi, P. and Zanini, M. G. (1982). Plasma and brain levels of glutamate and pyroglutamate after oral monosodium glutamate to rats. *Toxicol. Lett.,* 10, 169-175.

Chelius, D., Jing, K., Lueras, A., Rehder, D. S., Dillon, T. M., Vizel, A., Rajan, R. S., Li, T., Treuheit, M. J., and Bondarenko, P. V. (2006). Formation of pyroglutamic acid from N-terminal glutamic acid in immunoglobulin gamma antibodies. *Analytical chemistry*, 78(7), 2370-2376.

Connel, G. and Hanes, C. (1956). Enzymic Formation of Pyrrolidone Carboxylic Acid from γ-Glutamyl Peptides. *Nature,* 177, 377 – 378.

Cortacero-Ramírez, S., Segura-Carretero, A., Hernáinz-Bermúdez de Castro, M., and Fernández-Gutiérrez, A. (2005). Determination of low-molecular-mass organic acids in any type of beer samples by coelectroosmotic capillary electrophoresis. *Journal of Chromatography A*, 1064(1), 115 – 119.

Drago, F., Continella, G., Valerio, C. et al. (1987). Effects on pyroglutamic acid on learning and memory processes of the rat. *Acta Therapeutica*, 13, 587 – 594.

Drago, F., Valerio, C., D'Agata, V., Astuto, C., Spadaro, F., Continella, G. and Scapagnini, U. (1998). Pyroglutamic acid improves learning and memory cacapicties in old rats. *Funct. Neurol*., 3, 137 – 143

Duncan, S. H., Flint, H. J., Barcenilla, A., Stewart, C. S., & Hold, G. L. (2002). Roseburia intestinalis sp. nov., a novel saccharolytic, butyrate-producing bacterium from human faeces. *International Journal of Systematic and Evolutionary Microbiology*, 52(5), 1615–1620.

Duncan, S. H., Holtrop, G., Lobley, G. E., Calder, A. G., Stewart, C. S., & Flint, H. J. (2004). Contribution of acetate to butyrate formation by human faecal bacteria. *British Journal of Nutrition,* 91(6), 915-923.

Dusticier, N., Kerkerian L., Errami, M. and Nieoullon, A. (1985). Effects of pyroglutamic acid on corticostriatal glutamateric transmission. *Neuropharmacology*, 24, 903 – 908.

Fietzek, P. P., Breitkreutz, D. and Kuhn, K. (1974). Amino acid sequence of the amino-terminal region of calf skin collagen. *Biochim. Biophys. Acta*, 365, 305 – 310.

Gazme, B., Boachie, R. T., Tsopmo, A., and Udenigwe, C. C. (2019). Occurrence, properties and biological significance of pyroglutamyl peptides derived from different food sources. *Food Science and Human Wellness*.

Gibson, W. T., Scott, I. R. (1992). Cosmetic composition comprising an ester of pyroglutamic acid. US patentNo. 5,158,955.

Godshall, M. A., Buchler, I. P., Boyle, H. G., and Clarke, M. A. (1988). Changes in white sugars under accelerated storage conditions. *Sugar Processing Research Institute*, 400 – 408.

Gonzales, T., Robert-Baudouy, J. (1996). Bacterial aminopeptidases: Properties and functions, *FEMS Microbiology Reviews*, 18(4), 319 – 344.

Grioli, S., Lomeo, C., Quattropani, M. C., Spignoli, G., Villardita, C. (1990). Memory deficits in elderly people: Evaluation of treatment with pyroglutamic acid. *Fund Clin Pharmacol.*

Haefely, W., Pieri L., Polc, P. and Schaffner, R. (1981). General pharmacology and neuropharmacology of benzodiazepine derivatives. *Handb. Exp. Pharm*., 55, 13-262

Haitinger, L. (1882). Vorlauge Mittheilung iiber Glutaminsaure und Pyrrol. Monatsh. Chem., 3, 228-229.

Hamer, H. M., Jonkers, D. M. A. E., Venema, K., Vanhoutvin, S. A. L. W., Troost, F. J., & Brummer, R. J. (2008). The role of butyrate on colonic function. *Alimentary pharmacology & therapeutics*, 27(2), 104-119.

Hawkins, R. A., Simpson, I. A., Mokashi, A., Vina, J. R. (2006). Pyroglutamate stimulates Na+-dependent glutamate transport across the blood-brain barrier. *FEBS Lett*, 580, 4382–4386.

Hotamisligil, G. S., Shargill, N.S., and Spiegelman, B. M. (1993). Adipose expression of tumor necrosis factor-a: direct role in obesity-linked insulin resistance. *Science*, 259, 87 – 91.

Inoue, S., Okita, Y., de Toledo, A., Miyazaki, H., Hirano, E., and Morinaga, T. (2015). Pyroglutamic acid stimulates DNA synthesis in rat primary hepatocytes through the mitogen-activated protein kinase pathway. *Bioscience, Biotechnology, and Biochemistry*, 79(5), 795 – 798.

Jose, A., Takeru, H. (1991). Pyroglutamic Acid Esters Used as Dermal Penetration Enhancers for Drugs. US Patent No. 5,066,648.

Kaneko, K., Tsuji, K., Kim, C. H., Otoguro, C., Sumino, T., Aida, K., Sahara, K. and Kaneda, T. (1994). Contents and compositions of free sugars, organic acids, free amino acids and oligopeptides in soy sauce and soy paste

produced in Korea and Japan. *Nippon Shokuhin Kogyo Gakkaishi*, 41(2), 148 – 156.

Kimura, Y., Kido, T., Takaku, T., Sumiyoshi, M., Baba, K. (2004). Isolation of an anti- angiogenic substance from *Agaricus blazei* Murill: its antitumor and antimetastatic actions. *Cancer Sci*., 95, 758 – 764.

Knudsen, K. E. B., Serena, A., Canibe, N., & Juntunen, K. S. (2003). New insight into butyrate metabolism. *Proceedings of the Nutrition Society*, 62(1), 81-86.

Kouge, T., Yamashita, A., Ishihara, K. (2011). Method for angiogenesis inhibition or immunostimulation. US Patent No. 7,977,379 B2

Krishnaswamy, P. R., Pamiljans, V., Meister, A. (1960). Activated glutamate intermediate in the enzymatic synthesis of glutamine. *Journal of Biological Chemistry*, 235, PC39 – PC40.

Kumar, A., and Bachhawat, A. K. (2010). OXP1/YKL215c encodes an ATP-dependent 5-oxoprolinase in *Saccharomyces cerevisiae*: functional characterization, domain structure and identification of actin-like ATP-binding motifs in eukaryotic 5-oxoprolinases. *FEMS yeast research*, 10(4), 394 – 401

Kumar, A., & Bachhawat, A. K. (2012). Pyroglutamic acid: throwing light on a lightly studied metabolite. *Current Science*, 288-297.

Lake, M. A. (2020). What we know so far: COVID-19 current clinical knowledge and research. *Clinical Medicine*, 20(2), 124.

Lemieux, L., and Simard, R. E. (1992). Bitter flavour in dairy products. II. A review of bitter peptides from caseins: their formation, isolation and identification, structure masking and inhibition. *Lait*, 72, 335 – 382.

Liu, K. X., Okazawa, I., Kaku, T. (1995). Effect of Laennec on Liver Regeneration. *Clin. Pharmacol. Ther*., 5, 2187 – 2194.

Liu, Y. C., Chen, M. J., and Lin, C. W. (2002). Studies on Lao-Chao culture filtrate for a flavoring agent in a yogurt-like product. *Asian-australasian journal of animal sciences*, 15(4), 602 – 609.

Liu, L., Cao, Y., Chen, C., Zhang, X., McNabola, A., Wilkie, D., ... & Carter, C. (2006). Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5. *Cancer research*, 66(24), 11851 – 11858.

Liu, Y. D., Goetze, A. M., Bass, R. B., & Flynn, G. C. (2011). N-terminal glutamate to pyroglutamate conversion in vivo for human IgG2 antibodies. *Journal of Biological Chemistry*, 286(13), 11211 – 11217.

Louis, P., & Flint, H. J. (2009). Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. *FEMS microbiology letters,* 294(1), 1-8.

Macfarlane, G. T., & Macfarlane, S. (2012). Bacteria, Colonic Fermentation, and Gastrointestinal Health. *Journal of AOAC International*, 95(1), 50–60.

Maeso, N., del Castillo, C., Cornejo, L., García-Acicollar, M., Alguacil, L. F., and Barbas, C. (2006). Capillary electrophoresis for caffeine and pyroglutamate determination in coffees. *Journal of Pharmaceutical and Biomedical Analysis,* 41(4), 1095 – 1100.

Marconi, O., Floridi, S., and Montanari, L. (2007). Organic acids profile in tomato juice by HPLC with UV detection. *Journal of food quality*, 30(2), 253 – 266.

Marstein, S., Jellum, E., and Eldjarn, L. (1973). The concentration of pyroglutamic acid (2-pyrrolidone-5-carboxylic acid) in normal and psoriatic epidermis, determined on a microgram scale by gas chromatography. *Clinica Chimica Acta*, 49(3), 389 – 395.

Masaaki, N., Akio, S., Manabu, I., and Hitomi, Y. (1992). Comparision of organic acid patterns in miso. *Shishu Miso Kenkyosho Kenkyu Hokoku*, 33, 58 – 63

Meister, A. (1973). On the enzymology of amino acid transport. *Science*, 180, 33 – 39

Miyauchi, S., Gopal, E., Babu, E., Srinivas, S. R., Kubo, Y., Umapathy, N. S., Thakkar, V. S., Ganapathy, V and Prasad, P. D. (2010). Sodium-coupled electrogenic transport of pyroglutamate (5-oxoproline) via SLC5A8, a monocarboxylate transporter. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1798*(6), 1164 – 1171.

Moret, C. & Briley, M. (1988). The 'forgotten' amino acid pyroglutamate. *Trends Pharmacol.Sci*., 9, 278 – 279.

Mucchetti, G., Locci, F., Gatti, M., Neviani, E., Addeo, F., Dossena, A., and Marchelli, R. (2000). Pyroglutamic Acid in Cheese: Presence, Origin, and Correlation with Ripening Time of Grana Padano Cheese. *Journal of Dairy Science*, 83(4), 659 – 665.

Mucchetti, G., Locci, F., Massara, P., Vitale, R., and Neviani, E. (2002). Production of Pyroglutamic Acid by Thermophilic Lactic Acid Bacteria in Hard-cooked Mini- Cheeses. *Journal of Dairy Science*, 85(10), 2489 – 2496.

Murgia, A., Scano, P., Contu, M., Ibba, I., Altea, M., Bussu, M., Demuru, M., Porcu, A. and Caboni, P. (2016). Characterization of donkey milk and metabolite profile comparison with human milk and formula milk. *LWT*, 74, 427 – 433.

Nájera, C., Yus, M. (1999). Pyroglutamic acid: a versatile building block in asymmetric synthesis. *Tetrahedron: Asimmetry*, 10, 2245 – 2303.

Nishimura, A., Oyama, H., Hamada, T., Nobuoka, K., Shin, T., Murao, S. and Oda, K. (2000). Molecular cloning, sequencing, and expression in Escherichia coli of the gene encoding a novel 5-oxoprolinase without ATP-hydrolyzing activity from *Alcaligenes faecalis* N-38°. *Appl.Environ. Microbiol.*, 66, 3201 – 3205.

Orlowski, M., Meister, A. (1973). Glutamyl cyclotransferase. Distribution, isozymic forms, and specificity. *Journal of Biological Chemistry*, 248(8), 2836 – 2844.

Panari, G. (1985). L'acido lattico e l'acido piroglutammico nella maturazione del formaggio Parmigiano-Reggiano. *Sci. Tecn. Latt. Casear*., 36, 98 – 109.

Perlman, J. H., Thaw, C. N., Laakkonen, L., Bowers, C. Y., Osman, R. and Gershengorn, M. C. (1994). Hydrogen bonding interaction of thyrotropin-releasing hormone (TRH) with transmembrane tyrosine 106 of the TRH receptor. *Journal of Biology Chemistry*, 269, 1610 – 1613.

Petersen, J. S., Fels, G., and Rapoport, H. (1984). Chirospecific syntheses of (+) – and (-) -anatoxin a. *Journal of the American Chemical Society*, 106(16), 4539 – 4547.

Pfeiffer, P., König, H. (2009). Pyroglutamic Acid: A Novel Compound in Wines. In: König H., Unden G., Fröhlich J. (eds) Biology of Microorganisms on Grapes, in Must and in Wine, Springer, Berlin, Heidelberg.

Ristoff, E., and Larsson, A. (2007). Inborn errors in the metabolism of glutathione. *Orphanet J. Rare Dis*., 2, 16.

Schilling, S., Zeitschel, U., Hoffmann, T., Heiser, U., Francke, M., Kehlen, A., ... & Jagla, W. (2008). Glutaminyl cyclase inhibition attenuates

pyroglutamate Aβ and Alzheimer's disease–like pathology. *Nature medicine*, 14(10), 1106.

Seddon, A. P. and Meister, A. (1986). Trapping of an intermediate in the reaction catalyzed by 5-oxoprolinase. *Journal of Biology Chemistry*, 261, 11538 – 11543.

Sinforiani, E., Trucco, M., Cavallini, A., Gualtieri, S., Verri, A. P., Spignoli, G. (1985). Sulla reversibilità dei disordini cognitivi negli alcoolisti cronici in fase di assuefazione. Effetti del piroglutammato di arginina. *Min Psichiat,* 26, 339 – 342

Smith, K. T., & Workman, J. L. (2009). Histone deacetylase inhibitors: anticancer compounds. *The international journal of biochemistry & cell biology*, 41(1), 21-25.

Suganami, T., Nishida, J. and Ogawa, Y. (2005). A paracrine loop between adipocytes and macrophages aggravates inflammatory changes: role of free fatty acids and tumor necrosis factor a. *Arterioscler Thromb Vasc Biol*., 25, 2062–2068.

Syuhei, K., Hiroshi, T., and Hiroshi, I. (1994). The improvement and development of Chinese soy sauce: 1. The chemical composition of Chinese soy sauce. *Nippon Shoyu Kenkyusho Zasshi,* 20, 1

Terry, E. A. & Chang, Y. L. (1975). Kinetic study of the cyclization of L-glutamine to 2-pyrrolidone-5-carboxylic acid in a model system. *Journal of Agricultural and Food Chemistry*, 23(4), 828 – 830.

Uhlhaas, S., Lange, H. (1988). Striatal deficiency of L-pyroglutamic acid in Huntingdon's disease is accompanied by increased plasma levels. *Brains Res,* 457, 196 – 199.

Van Der Werf, P., Meister, A. (1975). The metabolic formation and utilization of – oxo-L-proline (L-pyroglutamate, L-pyrrolidone carboxylate). *Adv Enzymol,* 43, 519 – 566.

Venema, K. (2010). Role of gut microbiota in the control of energy and carbohydrate metabolism. *Current Opinion in Clinical Nutrition & Metabolic Care,* 13(4), 432-438.

Vorwerk, C. K., Naskar, R., Schuettauf, F., Quinto, K., Zurakowski, D., Gochenauer, G., Robinson, M.B., Mackler , S.A., Dreyer, E.B. (2000). Depression of retinal glutamate transporter function leads to elevated

intravitreal glutamate levels and ganglion cell death. *Invest Ophthalmol Vis Sci,* 41, 3615 – 3621.

Xydakis, M. S., Dehgani-Mobaraki, P., Holbrook, E. H., Geisthoff, U. W., Bauer, C., Hautefort, C., ... & Hopkins, C. (2020). Smell and taste dysfunction in patients with COVID-19. The Lancet Infectious Diseases, 20(9), 1015-1016.

Yang, Z., Suomalainen, T., Mäyrä-Mäkinen, A., and Huttunen, E. (1997). Antimicrobial Activity of 2-Pyrrolidone-5-Carboxylic Acid Produced by Lactic Acid Bacteria. *Journal of Food Protection*, 60(7), 786 – 790.

Yeom, H. W., Streaker, C. B., Zhang, Q. H., and Min, D. B. (2000). Effects of pulsed electric fields on the quality of orange juice and comparison with heat pasteurization. *Journal of Agricultural and Food Chemistry,* 48, 4597 – 4605.

Yoshinari, O., & Igarashi, K. (2011). Anti-diabetic effect of pyroglutamic acid in type 2 diabetic Goto-Kakizaki rats and KK-Ay mice. *British Journal of Nutrition,* 106(7), 995-1004.

Wang, Y., Wang, Y., Chen, Y., & Qin, Q. (2020). Unique epidemiological and clinical features of the emerging 2019 novel coronavirus pneumonia (COVID-19) implicate special control measures. *Journal of medical virology,* 92(6), 568-576.

Wegener, S., Kaufmann, M., and Kroh, L. W. (2017). Influence of l-pyroglutamic acid on the color formation process of non-enzymatic browning reactions. *Food Chemistry*, *232*, 450 – 454.

Willemsen, L. E. M., Koetsier, M. A., Van Deventer, S. J. H., & Van Tol, E. A. F. (2003). Short chain fatty acids stimulate epithelial mucin 2 expression through differential effects on prostaglandin E1 and E2 production by intestinal myofibroblasts. *Gut,* 52(10), 1442-1447.

Wirths, O., Breyhan, H., Cynis, H., Schilling, S., Demuth, H. U. and Bayer, T. A. (2009). Intraneuronal pyroglutamate- Abeta 3-42 triggers neurodegeneration and lethal neurological deficits in a transgenic mouse model. *Acta Neuropathol*., 118, 487 – 496.

Zhang, X., Luhrs, K. J., Ryff, K. A., Malik, W. T., Driscoll, M. J., & Culver, B. (2009). Suppression of nuclear factor kappa B ameliorates astrogliosis but not amyloid burden in APPswe/PS1dE9 mice. *Neuroscience,* 161(1), 53-58.

# CHAPTER 2. 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. In this study, the presence and kinetics of pGlu formation in fermented milk products were investigated from a chemical point of view. The pGlu was quantified in different fermented milk samples, especially yoghurt and kefir. 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 milk ranged from 51.65 to 277.37 mg 100 $g^{-1}$ of 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 $g^{-1}$ of 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 $g^{-1}$ of dry matter after 30 days.

**Key words:** pidolic acid; 5-oxoproline; plain yogurt; Greek yogurt, kefir; high-performance liquid chromatography

## 2.1 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 cyclizes 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 *et al.*, 2004**), mitogenic (**Inoue *et al.*, 2015; Oono *et al.*, 2009**), anxiolytic (**Sinforiani *et al.*, 1985; Antonelli *et al.*, 1984**), 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 *et al.*, 2020**), plasma, and cerebrospinal fluids (**Eckstein, Ammerman *et al.*, 2008**). In food, pGlu is found both as an amino residue in proteins or pyroglutamic peptides and 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 (**Kumar & Bachhawat, 2012; Kiyono *et al*., 2013**). 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 *et al.,* 2019**), Japanese rice wine (**Kiyono *et al.,* 2013**) and dry-cured ham (**Paolella *et al.,* 2018**). **Sforza *et al.*** (**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 *et al.,* 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 (**Mucchetti *et al.,* 2000**) since it is a parameter reasonably related to the ripening time (**Masotti *et al.,* 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 cyclization of glutamine.

In fermented food, such as ripened cheese (**Mucchetti *et al*., 2000**) and Zimbabwean naturally fermented milk (**Gadaga, *et al.,* 2001**), it has also been hypothesized 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*, *L. delbrueckii* subsp. *lactis* and *Streptococcus thermophilus* (**Mucchetti *et al.,* 2000; Mucchetti *et al.*, 2002**). **Murgia *et al.,* (2019**) found pGlu in yogurts 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 yogurt 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 aims to quantify pGlu in different fermented milk types - yogurt, kefir and other

probiotic fermented milk products available on the market and to define the kinetics of pGlu formation to increase knowledge on the chemical composition of fermented milk.

## 2.2 MATERIALS AND METHODS

### *2.2.1 Materials*

Different commercially available yogurt (LFY1, LFY2, LFY3, WY1, WY2, WY3, LFGY1, LFGY2, WGY1, WGY2) and fermented milk (PFM1, PFM2, PFM3, PFM4, PFM5, FFM1, FFM2, K1, K2, K3) types were purchased from a local market and stored at -18 °C until analysis. Three production batches for each type of sample were purchased. The category, type, protein percentage, fat percentage and microbial composition reported on the label of each product are shown in **Table 1.1.**

Laboratory-scale yogurt was produced in triplicate using 1000 mL of commercial ultra high temperature (UHT) sterilized whole milk (3.4% protein, 3.6% fat and 5% lactose) and inoculating with a blend of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (1:1 ratio, Yogurt 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 five hours (when the initial pH value of 6.6 had dropped to 4.3). A sample of UHT-sterilized whole milk without addition of microorganisms was incubated under the same conditions for five hours and used as a control. Then, the set yogurt 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.

### *2.2.2 Chemicals*

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

### *2.2.3 pH determination*

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

### *2.2.4 Moisture content*

Representative samples (~3 g each) of the yogurt 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 (d.m.) could be determined gravimetrically (**Koc *et al.,* 2010**).

#### *2.2.5 HPLC determination of sugar content*

Sugar extraction was performed by dissolving one gram of yogurt sample in 10 mL of 0.5 M sulphuric acid, and centrifuging at 10000 g for 10 min (**Wang *et al.* 2010**). The supernatant was collected and filtered with a 0.45μm filter.

The glucose, galactose and lactose contents were determined by injecting twenty microlitres of 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 x 250 mm, 5 □m Agilent Technologies Inc.) was used. The calibration curves were constructed with glucose, galactose and lactose standard solutions (2500; 5000; 10000 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 $mL^{-1}$ of sample).

### *2.2.6 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 & Califano (1989)**, with some modifications. Approximately 3.5 g of each sample was added to 25 mL of aqueous buffer $(NH_4)_2HPO_4$ 0.5% w/w in bidistilled water, stirred for 1 min, extracted for 1 hour, 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 x 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 **Figure 1.1**, where the most abundant peaks were pGlu and lactic acid.

### *2.2.7 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).

## 2.3 RESULTS AND DISCUSSION

### *2.3.1 pH and lactic acid content of commercial yogurt and fermented milk*

Acidity is one of the major indices for consumers' acceptability of plain yogurt 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 4.47 in the fermented probiotic milk PFM1 and the whole Greek yogurt WGY1, respectively (**Table 2.2**), in line with the range of 4.00 – 4.60 reported by different literature sources (**Al-Kadamany *et al.,* 2007**). In particular **Aryana & McGrew, 2007** found mean pH values ranging from 4.32 to 4.60 in yogurt with *Lacticaseibacillus casei* and various prebiotics; **Al-Kadamany *et al.* (2003)** found a pH range of 3.47 – 4.05 in concentrated yogurt during shelf life. **Wang *et al.* (2010)** found that pH values of yogurts inoculated with *L. 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 yogurt a sharp and acidic taste. The concentration of lactic acid varies from 0.04-0.3% in fermented milk (**Aiello *et al.,* 2019**) to 0.8-1.3% in yogurt (**Liu & Lv, 2019**), mainly depending on the microbial cultures and the conditions of time and temperature of the fermentation (**Abdel-Rahman *et al.,* 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 analysed samples (**Table 2.2**), 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 yogurts, **Vénica *et al.,* (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, **Guzem-Seydim *et al.,* (1999**) showed a lower concentration that ranged from 4.57% at time 0 of storage to 5.50% after 21 days of storage, while **Gronnevik *et al.*(2011**) found a concentration of lactic acid of 5.71% after 8 weeks of storage at 5.5-6 °C.

### *2.3.2 pGlu content in commercial yogurt and fermented milk*

**Table 2.2** shows the pGlu contents in all samples of each category of fermented milk. LFY3 showed the highest pyroglutamic content (277.37 mg 100 $g^{-1}$ d.m.), while PFM1 had the lowest (51.65 mg 100 $g^{-1}$ d.m.). Low-fat yogurt (LFY) exhibited the highest content of pyroglutamic acid (200.22 – 277.33 mg 100 $g^{-1}$ d.m.), while whole Greek yogurt (WGY) showed the lowest concentration (106.14 - 146.04 mg 100 $g^{-1}$ d.m.).

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

The presence of pGlu in fermented milk and yogurt may depend on the cyclase activity of thermophilic lactic acid bacteria (especially *Streptococcus thermophilus* and *Lactobacillus 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 *et al.* (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 *et al.* (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.

### *2.3.3 Assessments of pH, lactic acid, moisture and sugars in laboratory-scale yoghurt*

Usually, in the manufacture of yoghurt, the heated milk is conventionally sterilized at 82 -90 °C for 5-30 minutes 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, sterilized milk is particularly suitable due to the formation of insoluble adducts of β-lactoglobulin and κ-casein at high temperatures (**Verruck *et al.*, 2019**) and the formation of a stable clot that is not subject to syneresis (**Alais, 2000**). The milk used in this work was UHT-sterilized, as several reports have shown that UHT has many possible advantages over the conventional

method of sterilization, including better process control and sanitation, energy and time savings, high microbial quality, longer shelf life of the product and the stimulation of growth and activity of yoghurt cultures (**Krasaekoopt *et al.*, 2003**).

Yoghurt formation occurred five hours after the addition of the microorganisms, that is, when the pH of milk changed from the initial value of 6.6 to 4.4 (**Figure 2.2a**). 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 *et al.*(2003**), is linked to the increase in lactic acid, which ranged from 0.38 to 4.33% in the d.m. (**Figure 2.2a**). The pH and lactic acid trend in yogurt during 30 days of storage is shown in **Figure 2.2b**. 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 the yogurt was 87.90%. **Mahmood *et al.***(2008) showed that the moisture content in plain yoghurt prepared with buffalo milk was 80.47%; **Curti, *et al.*,** (**2017**) showed a concentration of 82.60% in yoghurt prepared with ultrapasteurized milk, while **Sahan Yasar & Hayaloglu** (**2008**) showed a concentration that ranged from 86.62% to 87.21% of moisture in no-fat yoghurts prepared with different contents of beta-glucans. Furthermore, **Vénica et al.** (**2014**) showed a moisture content of 88% for natural yoghurt and 82% for sweetened yoghurt.

To characterize 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 five-hour incubation at 40 °C and the 30-day storage at 4 °C in uninoculated milk (**Figure 2.3a**, **2.3b**). In the inoculated milk, the lactose content decreased during fermentation to 4.1% (decrease of approximately 16%) after 5 hours, when clot formation occurred (**Figure 2.3a**) and to 2.4% (final decrease of 39%) after 30 days of storage (**Figure 2.3b**). 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 (**Figure 2.3a**, **2.3b**). 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 *L. casei* Zhang after 21 days of storage and that galactose increased. The results are also in agreement with those of **Delgado-Fernández *et al.* (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 yogurt*

**Figure 2.4a** shows the kinetics of pGlu formation in milk during fermentation at 40 °C to produce yogurt and in the uninoculated control milk. Surprisingly, pGlu was already present in UHT-sterilized milk at the beginning of fermentation (188.47 mg 100 $g^{-1}$ d.m.). This could be due to the spontaneous conversion of glutamine into pGlu by the loss of a water molecule at high temperatures (**Kumar & Bachhawat, 2012**) during the sterilization treatment carried out on the milk. After five hours of fermentation, pGlu slightly decreased (from 188.47 to 174.74 mg 100 $g^{-1}$ d.m.) but not in a statistically significant way (**Figure 2.4a**). The same trend was observed by **Mugula *et al.* (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 an 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 $g^{-1}$ d.m. at 30 days (**Figure 2.4b**). The pGlu concentration was constant at 190.10 – 200.96 mg 100 $g^{-1}$ d.m. 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 to the control. This increase is related to the starter microflora rather than to the raw milk.

Indeed, it is known that yoghurt bacteria (*S. thermophilus* and *L. 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 cyclized 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 *et al.***(**2002**) for a traditional Chinese fermented rice product. In particular, enzymes responsible for this cyclization could be glutamate 5-kinase, identified in *Streptococcus thermophilus* by **Massarelli *et al.*** (**2000**), which catalyses the phosphorylation of glutamate which becomes highly unstable and prone to spontaneous cyclization into pyroglutamic acid (**Kumar & Bachhawat**, **2012**), or glutamine cyclase, which is present in both *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus,* as reported by **Mucchetti *et al.*** (**2002**).

## 2.4 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 $g^{-1}$ d.m. as a function of the thermophilic lactic acid bacteria used as starter cultures.

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

These findings are also interesting because pGlu in food could have beneficial effects (antitumoural, mitogenic, anxiolytic, anti-diabetic 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.

**References**


Abdel-Rahman, M. A., Tashiro, Y., & Sonomoto, K. (2013). Recent advances in lactic acid production by microbial fermentation processes. *Biotechnology advances*, *31*(6), 877-902. https://doi.org/10.1016/j.biotechadv.2013.04.002

Adamberg, K., Kask, S., Laht, T. M., & Paalme, T. (2003). The effect of temperature and pH on the growth of lactic acid bacteria: a pH-auxostat study. International *Journal of Food Microbiology*, 85(1-2), 171-183. https://doi.org/10.1016/S0168-1605(02)00537-8.

Aiello, A., Pizzolongo, F., Manzo, N., & Romano, R. (2019). A new method to distinguish milk adulteration with neutralizers by detection of lactic acid. *Food Analytical Methods*, 12(11), 2555-2561. https://doi.org/10.1007/s12161-019-01594-5

Al-Kadamany, E., Khattar, M., Haddad, T., & Toufeili, I. (2003). Estimation of shelf-life of concentrated yogurt by monitoring selected microbiological and physicochemical changes during storage. *LWT-Food Science and Technology*, *36*(4), 407-414. https://doi.org/10.1016/S0023-6438(03)00018-5

Alais, C. (2000). Scienza del latte: principi di tecnologia del latte e dei derivati (3rd ed.).Milano, Italy. Ed: Tecniche Nuove.

Antonelli, T., Carla, V., Lambertini, L., Moroni, F., & Bianchi, C. (1984). Pyroglutamic acid administration modifies the electrocorticogram and increases the release of acetylcholine and GABA from the guinea-pig cerebral cortex. *Pharmacological research communications*, *16*(2), 189-197.https://doi.org/10.1016/S0031-6989(84)80094-6

Aryana, K. J., & McGrew, P. (2007). Quality attributes of yogurt with Lactobacillus casei and various prebiotics. *LWT-Food Science and Technology*, 40(10), 1808-1814. https://doi.org/10.1016/j.lwt.2007.01.008

Bevilacqua, A. E., & Califano, A. N. (1989). Determination of organic acids in dairy products by high performance liquid chromatography. *Journal of Food Science*, *54*(4), 1076-1076. https://doi.org/10.1111/j.1365-2621.1989.tb07948.x

Careri, M., Spagnoli, S., Panari, G., Zannoni, M., & Barbieri, G. (1996). Chemical parameters of the non-volatile fraction of ripened Parmigiano-Reggiano cheese. *International Dairy Journal*, 6(2), 147-155. https://doi.org/10.1016/0958-6946(94)00056-5

Casolari, A. (2007). Spallanzani e Pasteur. Ed: Lampi di stampa, Milano, Italy.

Curti, C. A., Vidal, P. M., Curti, R. N., & Ramón, A. N. (2017). Chemical characterization, texture and consumer acceptability of yogurts supplemented with quinoa flour. *Food Science and Technology*, 37, 627-631. https://doi.org/10.1590/1678-457X.27716

Delgado-Fernández, P., Corzo, N., Olano, A., Hernández-Hernández, O., & Moreno, F. J. (2019). Effect of selected prebiotics on the growth of lactic acid bacteria and physicochemical properties of yogurts. *International Dairy Journal*, 89, 77-85. https://doi.org/10.1016/j.idairyj.2018.09.003

Delgado-Fernández, P., Moreno, F. J., Olano A., Hernández-Hernández, O., Olano, A., Moreno, F. J., & Corzo, N. (2020). Probiotic viability in yoghurts containing oligosaccharides derived from lactulose (OsLu) during fermentation and cold storage. *International Dairy Journal*, 102, 104621, 1-7. http://dx.doi.org/10.1016/j.idairyj.2019.104621

Eckstein, J. A., Ammerman, G. M., Reveles, J. M., & Ackermann, B. L. (2008). Analysis of glutamine, glutamate, pyroglutamate, and GABA in cerebrospinal fluid using ion pairing HPLC with positive electrospray LC/MS/MS. *Journal of Neuroscience Methods*, 171(2), 190-196. https://doi.org/10.1016/j.jneumeth.2008.02.019

Forgacsova, A., Galba, J., Garruto, R. M., Majerova, P., Katina, S., & Kovac, A. (2018). A novel liquid chromatography/mass spectrometry method for determination of neurotransmitters in brain tissue: Application to human tauopathies. *Journal of Chromatography B*, 1073, 154-162. https://doi.org/10.1016/j.jchromb.2017.12.015

Gadaga, T. H., Mutukumira, A. N., & Narvhus, J. A. (2001). The growth and interaction of yeasts and lactic acid bacteria isolated from Zimbabwean naturally fermented milk in UHT milk. International *Journal of Food Microbiology*, 68(1-2), 21-32. https://doi.org/10.1016/s0168-1605(01)00466-4

Gazme, B., Boachie, R. T., Tsopmo, A., & Udenigwe, C. C. (2019). Occurrence, properties and biological significance of pyroglutamyl peptides derived from different food sources. *Food Science and Human Wellness*, 8(3), 268-274. https://doi.org/10.1016/j.fshw.2019.05.002

Grønnevik, H., Falstad, M., & Narvhus, J. A. (2011). Microbiological and chemical properties of Norwegian kefir during storage. *International Dairy Journal*, 21(9), 601-606. https://doi.org/10.1016/j.idairyj.2011.01.001

Guzel-Seydim, Z., Seydim, A. C., & Greene, A. K. (2000). Organic acids and volatile flavour components evolved during refrigerated storage of kefir. *Journal of Dairy Science*, 83(2), 275-277. https://doi.org/10.3168/jds.S0022-0302(00)74874-0

Higaki-Sato, N., Sato, K., Esumi, Y., Okumura, T., Yoshikawa, H., Tanaka-Kuwajima, C., Kurata, A., Koratu, M., Kawabata, M., Nakamura, Y., & Ohtsuki, K. (2003). Isolation and identification of indigestible pyroglutamyl peptides in an enzymatic hydrolysate of wheat gluten prepared on an industrial scale. *Journal of Agricultural and Food Chemistry*, 51(1), 8-13. https://doi.org/10.1021/jf020528i

Inoue, S., Okita, Y., de Toledo, A., Miyazaki, H., Hirano, E., & Morinaga, T. (2015). Pyroglutamic acid stimulates DNA synthesis in rat primary hepatocytes through the mitogen-activated protein kinase pathway. *Bioscience, Biotechnology, and Biochemistry*, 79(5), 795-798.https://doi.org/10.1080/09168451.2014.991689

Jiang, Y., Yang, C., Zheng, Y., Liu, Y., & Chen, Y. (2020). A set of global metabolomic biomarker candidates to predict the risk of dry eye disease. *Frontiers in Cell and Developmental Biology*, 8, 344. https://doi.org/10.3389/fcell.2020.00344.

Kimura, Y. (2005). New anticancer agents: in vitro and in vivo evaluation of the antitumor and antimetastatic actions of various compounds isolated from medicinal plants. *In Vivo*, 19(1), 37-60.

Kimura, Y., Kido, T., Takaku, T., Sumiyoshi, M., & Baba, K. (2004). Isolation of an anti-angiogenic substance from Agaricus blazei Murill: Its antitumor and antimetastatic actions. *Cancer Science*, 95(9), 758-764. https://doi.org/10.1111/j.1349-7006.2004.tb03258.x

Kiyono, T., Hirooka, K., Yamamoto, Y., Kuniishi, S., Ohtsuka, M., Kimura, S., Young Park, E.,Nakamura, Y., Sato, K. (2013). Identification of pyroglutamyl peptides in Japanese rice wine (sake): presence of hepatoprotective pyroGlu-Leu. *Journal of Agricultural and Food Chemistry*, 61(47), 11660-11667.https://doi.org/10.1021/jf404381w

Koc, B., Yilmazer, M. S., Balkır, P., & Ertekin, F. K. (2010). Spray drying of yogurt: Optimization of process conditions for improving viability and other quality attributes. *Drying Technology*, 28(4), 495-507.https://doi.org/10.1080/07373931003613809

Krasaekoopt, W., Bhandari, B., & Deeth, H. (2003). Yogurt from UHT milk: a review. *Australian Journal of Dairy Technology*, 58(1), 26.

Kumar, A., & Bachhawat, A. K. (2012). Pyroglutamic acid: throwing light on a lightly studied metabolite. *Current Science*, 288-297.

Liu, D., & Lv, X. X. (2019). Effect of blueberry flower pulp on sensory, physicochemical properties, lactic acid bacteria, and antioxidant activity of set?type yogurt during refrigeration. *Journal of Food Processing and Preservation*, 43(1), e13856. https://doi.org/10.1111/jfpp.13856

Liu, Y. C., Chen, M. J., & Lin, C. W. (2002). Studies on Lao-Chao culture filtrate for a flavoring agent in a yogurt-like product. *Asian-australasian Journal of Animal Sciences*, 15(4), 602-609.https://doi.org/10.5713/ajas.2002.602

Mahmood, A., Abbas, N., & Gilani, A. H. (2008). Quality of stirred buffalo milk yogurt blended with apple and banana fruits. *Pakistan Journal of Agricultural Sciences*, 45(2), 275-279.

Marconi, O., Floridi, S., & Montanari, L. (2007). Organic acids profile in tomato juice by HPLC with UV detection. *Journal of Food Quality*, 30(2), 253-266.https://doi.org/10.1111/j.1745-4557.2007.00119.x.

Masotti, F., Hogenboom, J. A., Rosi, V., De Noni, I., & Pellegrino, L. (2010). Proteolysis indices related to cheese ripening and typicalness in PDO Grana Padano cheese. *International Dairy Journal*, 20(5), 352-359. https://doi.org/10.1016/j.idairyj.2009.11.020

Massarelli, I., Forlani, G., Ricca, E., & De Felice, M. (2000). Enhanced and feedback-resistant γ-glutamyl kinase activity of an *Escherichia coli* transformant carrying a mutated proB gene of *Streptococcus thermophilus*. *FEMS Microbiology Letters*, *182*(1), 143-147. https://doi.org/10.1111/j.1574-6968.2000.tb08888.x

Mucchetti, G., Locci, F., Gatti, M., Neviani, E., Addeo, F., Dossena, A., & Marchelli, R. (2000). Pyroglutamic acid in cheese: presence, origin, and correlation with ripening time of Grana Padano cheese. *Journal of Dairy Science*, 83(4), 659-665.https://doi.org/10.3168/jds.S0022-0302(00)74926-5

Mucchetti, G., Locci, F., Massara, P., Vitale, R., & Neviani, E. (2002). Production of pyroglutamic acid by thermophilic lactic acid bacteria in hard-cooked mini-cheeses. *Journal of Dairy Science*, 85(10), 2489-2496.https://doi.org/10.3168/jds.S0022-0302(02)74331-2

Mugula, J. K., Nnko, S. A. M., Narvhus, J. A., & Sørhaug, T. (2003). Microbiological and fermentation characteristics of togwa, a Tanzanian

fermented food. *International Journal of Food Microbiology*, 80(3), 187-199.https://doi.org/10.1016/S0168-1605(02)00141-1

Murgia, A., Scano, P., Cacciabue, R., Dessì, D., & Caboni, P. (2019). GC-MS metabolomics comparison of yoghurts from sheep's and goats' milk. *International Dairy Journal*, *96*, 44-49. https://doi.org/10.1016/j.idairyj.2019.03.012

Murgia, A., Scano, P., Contu, M., Ibba, I., Altea, M., Bussu, M., Demuru, M., Porcu, A., & Caboni, P. (2016). Characterization of donkey milk and metabolite profile comparison with human milk and formula milk. *LWT Food Science and Technology*, 74, 427-433. https://doi.org/10.1016/j.lwt.2016.07.070

Ochi, H., Sakai, Y., Koishihara, H., Abe, F., Bamba, T., & Fukusaki, E. (2013). Monitoring the ripening process of Cheddar cheese based on hydrophilic component profiling using gas chromatography-mass spectrometry. *Journal of Dairy Science*, 96(12), 7427-7441.http://dx.doi.org/ 10.3168/jds.2013-6897

Olsen, M. A., Vhile, S. G., Porcellato, D., Kidane, A., & Skeie, S. B. (2021). Feeding concentrates with different protein sources to high-yielding, mid-lactation Norwegian Red cows: Effect on cheese ripening. *Journal of Dairy Science*, 104(4), 4062-4073. https://doi.org/10.3168/jds.2020-19226

Oono, S., Kurimoto, T., Nakazawa, T., Miyoshi, T., Okamoto, N., Kashimoto, R., Tagami, Y., Ito, Y., & Mimura, O. (2009). Pyroglutamic acid promotes survival of retinal ganglion cells after optic nerve injury. *Current Eye Research*, 34(7), 598-605. https://doi.org/10.1080/02713680902981292

Paolella, S., Prandi, B., Falavigna, C., Buhler, S., Dossena, A., Sforza, S., & Galaverna, G. (2018). Occurrence of non-proteolytic amino acyl derivatives in dry-cured ham. *Food Research International*, 114, 38-46. https://doi.org/10.1016/j.foodres.2018.07.057

Pinto, G., Picariello, G., Addeo, F., Chianese, L., Scaloni, A., & Caira, S. (2020). Proteolysis and process-induced modifications in synbiotic yogurt investigated by peptidomics and phosphopeptidomics. *Journal of Agricultural and Food Chemistry*, 68(32), 8744-8754. https://doi.org/: 10.1021/acs.jafc.0c02603.

Sahan, N. U. R. A. Y., Yasar, K., & Hayaloglu, A. A. (2008). Physical, chemical and flavour quality of non-fat yogurt as affected by a β-glucan hydrocolloidal composite during storage. *Food Hydrocolloids*, 22(7), 1291-1297. https://doi.org/10.1016/j.foodhyd.2007.06.010

Salvadori del Prato, O. (2005). Tecnologie del latte. Burro, yogurt, gelato, latte alimentare. Pages: 269 – 334. Ed: Edagricole, Bologna, Italy.

Sinforiani, E., M. Trucco, A. Cavallini, S. Gualtieri, A. P. Verri, & G. Spignoli. 1985. Sulla reversibilità dei disordini cognitivi negli alcoolisti cronici in fase di assuefazione. Effetti del piroglutammato di arginina. Min Psichiat. 26: 339 – 342.

Sforza, S., Cavatorta, V., Galaverna, G., Dossena, A., & Marchelli, R. (2009). Accumulation of non-proteolytic aminoacyl derivatives in Parmigiano-Reggiano cheese during ripening. *International Dairy Journal*, 19(10), 582-587. https://doi.org/10.1016/j.idairyj.2009.04.009

Shihata, A., & Shah, N. P. (2000). Proteolytic profiles of yogurt and probiotic bacteria. *International Dairy Journal*, *10*(5-6), 401-408 https://doi.org/10.1016/S0958-6946(00)00072-8

Solano, F. (2020). Metabolism and functions of amino acids in the skin. *In Amino Acids in Nutrition and Health* (pp. 187-199). Springer, Cham.

Sperber, W. H. (1983). Influence of water activity on foodborne bacteria—a review. *Journal of Food Protection*, 46(2), 142-150.https://doi.org/10.4315/0362-028X-46.2.142

Vénica, C. I., Perotti, M. C., & Bergamini, C. V. (2014). Organic acids profiles in lactose-hydrolyzed yogurt with different matrix composition. *Dairy Science & Technology*, 94(6), 561-580. https://doi.org/10.1007/s13594-014-0180-7

Verruck, S., Sartor, S., & Marenda, F. B. (2019). Influence of heat treatment and microfiltration on the milk proteins properties. *Advances in Food Technology and Nutritional Sciences. Open Journal*, 5(2), 54-66. https://doi.org/10.17140/AFTNSOJ-5-157

Wang, J., Guo, Z., Zhang, Q., Yan, L., Chen, Y., Chen, X. I. A., Liu, X. M., Chen, W., & Zhang, H. P. (2010). Effect of probiotic Lactobacillus casei Zhang on fermentation characteristics of set yogurt. International *Journal of Dairy Technology*, 63(1), 105-112. https://doi: 10.1111/j.1471-0307.2009.00556.x

Wickham, H. 2011. ggplot2. Wiley Interdiscip Rev Comput Stat. 3(2): 180-185. https://doi.org/10.1002/wics.147

Yao, S., & Udenigwe, C. C. (2018). Peptidomics of potato protein hydrolysates: Implications of post-translational modifications in food

peptide structure and behaviour. *Royal Society Open Science*, 5(7), 172425. https://doi.org/10.1098/rsos.172425

Yoshinari, O., & Igarashi, K. (2011). Anti-diabetic effect of pyroglutamic acid in type 2 diabetic Goto-Kakizaki rats and KK-Ay mice. *British Journal of Nutrition*, 106(7), 995-1004. https://doi.org/10.1017/S0007114511001279

. **Table 2.1** Commercial specifications (category, type, protein and fat content) and microbial compositions of samples of low-fat yoghurt (LFY), whole yoghurt (WY), low-fat Greek yoghurt (LFGY), whole Greek yoghurt (WGY), probiotic fermented milk (PFM), functional fermented milk (FFM) and kefir (K) used in this study.

| Samples | Category | Type | Protein (%) | Fat (%) | Microbial composition |
|---|---|---|---|---|---|
| LFY1 | PLAIN YOGURT | LOW-FAT | 6 | 0 | *S. thermophilus; Lb. bulgaricus* |
| LFY2 | | | 5 | 0 | *S. thermophilus; Lb. bulgaricus* |
| LFY3 | | | 4 | 0 | *S. thermophilus; Lb. bulgaricus* |
| WY1 | | WHOLE | 4.8 | 3.7 | *S. thermophilus; Lb. bulgaricus* |
| WY2 | | | 3.7 | 4.2 | *S. thermophilus; Lb. bulgaricus* |
| WY3 | | | 3 | 3.4 | *S. thermophilus; Lb. bulgaricus* |
| LFGY1 | PLAIN GREEK YOGURT | LOW-FAT | 10 | 0 | *S. thermophilus; Lb. bulgaricus* |
| LFGY2 | | | 10 | 0 | *S. thermophilus; Lb. bulgaricus* |
| WGY1 | | WHOLE | 7.8 | 5 | *S. thermophilus; Lb. bulgaricus* |
| WGY2 | | | 9 | 5 | *S. thermophilus; Lb. bulgaricus* |
| PFM1 | FERMENTED MILK | PROBIOTIC | 1 | 0 | *Lb. casei Shirota* |
| PFM2 | | | 3.9 | 3.4 | *S. thermophilus; Lb. bulgaricus; Bifidobacterium spp.* |
| PFM3 | | | 3 | 1.6 | *Lb. paracasei subsp. paracasei CNCM I-3689; S. thermophilus; Lb. bulgaricus* |
| PFM4 | | | 2.9 | 0.9 | *Lb. casei Activ.* |
| PFM5 | | | 2.4 | 0.9 | *S. thermophilus; Lb. johnsonii La1* |
| FFM1 | | FUNCTIONAL (with 1.6% phytosterols) | 3.2 | 1.1 | *S. thermophilus; Lb. bulgaricus* |
| FFM2 | | | 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;* |

| | | | | | |
|---|---|---|---|---|---|
| K3 | | | 3.5 | 1.5 | *Lb. acidophilus; Lb. helveticus; Lb. lactis B. lactis; S. thermophilus Lc. lactis; Lc. cremoris; Lb. acidophilus; Lb. helveticus; Lb. lactis; B. lactis; S. thermophilus* |

** S.*: *Streptococcus*; *Lb.*: *Lactobacillus*; *Lc.*: *Lactococcus*; *B.*: *Bifidobacterium*

**Table 2.2** Values of pH, moisture, lactic acid and pyroglutamic acid (pGlu) content in commercial yoghurt and fermented milk: low-fat yoghurt (LFY), whole yoghurt (WY), low-fat Greek yoghurt (LFGY), whole Greek yoghurt (WGY), probiotic fermented milk (PFM), functional fermented milk (FFM) and kefir (K).

| Sample | pH | Moisture (%) | Lactic acid (% d.m.) | pGlu (mg 100 $g^{-1}$ d.m.) |
|---|---|---|---|---|
| LFY1 | 4.17 ± 0.01 $^{b, c, d}$ | 81.38 ± 0.11 $^{f, g, h, i}$ | 6.28 ± 0.11 $^{c, d, e, f}$ | 200.22 ± 25.48 $^{a, b, c, d}$ |
| LFY2 | 4.15 ± 0.01 $^{b, c, d}$ | 86.24 ± 0.34 $^{b, c, d}$ | 7.12 ± 0.21 $^{a, b, c, d}$ | 260.17 ± 31.51 $^{a, b}$ |
| LFY3 | 4.13 ± 0.01 $^{b, c, d, e}$ | 88.21 ± 0.25 $^{a, b, c}$ | 8.52 ± 0.12 $^{a}$ | 277.37 ± 48.68 $^{a}$ |
| WY1 | 4.17 ± 0.01 $^{b, c, d}$ | 83.93 ± 0.17 $^{d, e, f}$ | 4.16 ± 0.36 $^{h, i}$ | 192.23 ± 32.74 $^{b, c, d}$ |
| WY2 | 4.03 ± 0.01 $^{e, f}$ | 85.31 ± 0.34 $^{c, d, e}$ | 5.14 ± 0.29 $^{f, g, h}$ | 175.37 ± 33.34 $^{c, d, e}$ |
| WY3 | 3.85 ±0.01 $^{g}$ | 78.95 ± 0.68 $^{i}$ | 5.50 ± 0.67 $^{e, f, g, h}$ | 148.56 ± 20.99 $^{c, d, e}$ |
| LFGY1 | 4.20 ± 0.02 $^{b, c}$ | 82.04 ± 1.04 $^{f, g, h}$ | 5.25 ± 0.26 $^{f, g, h}$ | 142.95 ± 32.91 $^{d, e}$ |
| LFGY2 | 4.08 ± 0.02 $^{d, e, f}$ | 84.23 ± 0.20 $^{d, e, f}$ | 7.89 ± 0.60 $^{a, b}$ | 136.92 ± 8.54 $^{d, e}$ |
| WGY1 | 4.47 ± 0.01 $^{a}$ | 79.15 ± 0.01 $^{h, i}$ | 6.98 ± 0.19 $^{b, c, d, e}$ | 146.04 ± 8.27 $^{c, d, e}$ |
| WGY2 | 4.11 ± 0.01 $^{c, d, e, f}$ | 81.25 ± 0.48 $^{f, g, h, i}$ | 4.138 ± 0.68 $^{h, i}$ | 106.14 ± 30.48 $^{e, f}$ |
| PFM1 | 3.47 ± 0.01 $^{h}$ | 85.53 ± 0.89 $^{c, d}$ | 4.07 ± 0.96 $^{h, i}$ | 51.65 ± 12.80 $^{f}$ |
| PFM2 | 4.14 ± 0.01 $^{b, c, d}$ | 86.52 ± 0.23 $^{a, b, c, d}$ | 4.95 ± 0.34 $^{f, g, h, i}$ | 222.57 ± 79.56 $^{a, b, c}$ |
| PFM3 | 4.22 ± 0.02 $^{b}$ | 81.87 ± 0.87 $^{f, g, h, i}$ | 4.07 ± 0.14 $^{h, i}$ | 153.73 ± 13.43 $^{c, d, e}$ |
| PFM4 | 3.90 ± 0.01 $^{g}$ | 82.44 ± 0.01 $^{e, f, g}$ | 4.58 ± 0.31 $^{g, h, i}$ | 103.10 ± 30.28 $^{e, f}$ |
| PFM5 | 4.12 ± 0.01 $^{b, c, d, e}$ | 83.66 ± 0.67 $^{d, e, f}$ | 6.30 ± 0.23 $^{c, d, e, f}$ | 145.09 ± 9.35 $^{d, e}$ |
| FFM1 | 4.18 ±0.01 $^{b, c, d}$ | 85.53 ± 0.73 $^{a, b}$ | 4.65 ± 0.18 $^{g, h, i}$ | 152.28 ± 12.79 $^{c, d, e}$ |
| FFM2 | 4.01 ± 0.01 $^{f}$ | 80.23 ± 0.08 $^{g, h, i}$ | 3.50 ± 0.09 $^{i}$ | 126.97 ± 27.47 $^{e, f}$ |
| K1 | 4.16 ± 0.01 $^{b, c, d}$ | 83.61 ± 0.07 $^{d, e, f}$ | 5.84 ±0. 65 $^{d, e, f, g}$ | 161.09 ± 9.39 $^{c, d, e}$ |
| K2 | 4.20 ± 0.01 $^{b, c}$ | 84.06 ± 2.11 $^{d, e, f}$ | 6.04 ± 0.27 $^{c, d, e, f, g}$ | 166.52 ± 20.48 $^{c, d, e}$ |
| K3 | 4.09 ± 0.10 $^{d, e, f}$ | 89.50 ± 0.34 $^{a}$ | 7.51 ± 0.87 $^{a, b, c}$ | 253.29 ± 15.63 $^{a, b}$ |

* d.m.: dry matter;

$^{a-i}$ : Different letters in the same column indicate statistically significant differences ($p$ value < 0.05).

**Figure 2.1** Typical HPLC chromatogram of lactic (1) and pyroglutamic (2) acids.

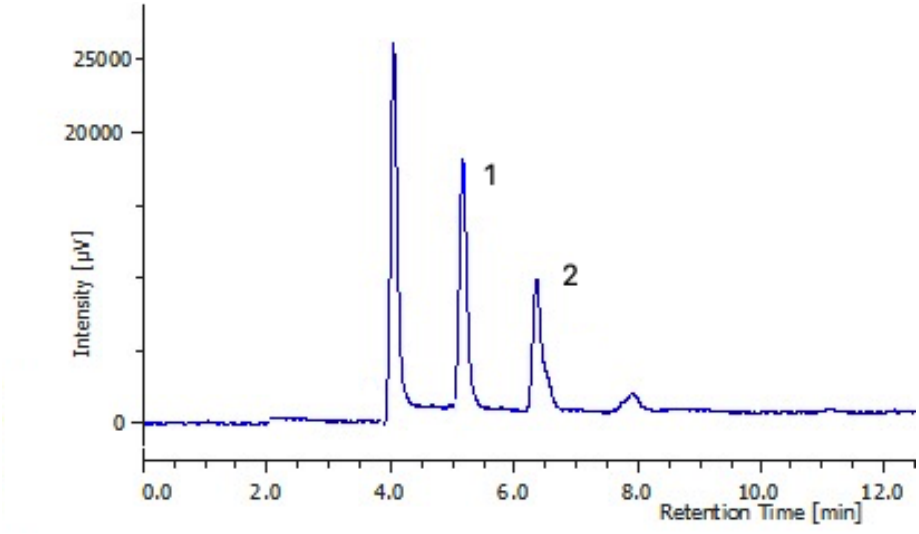
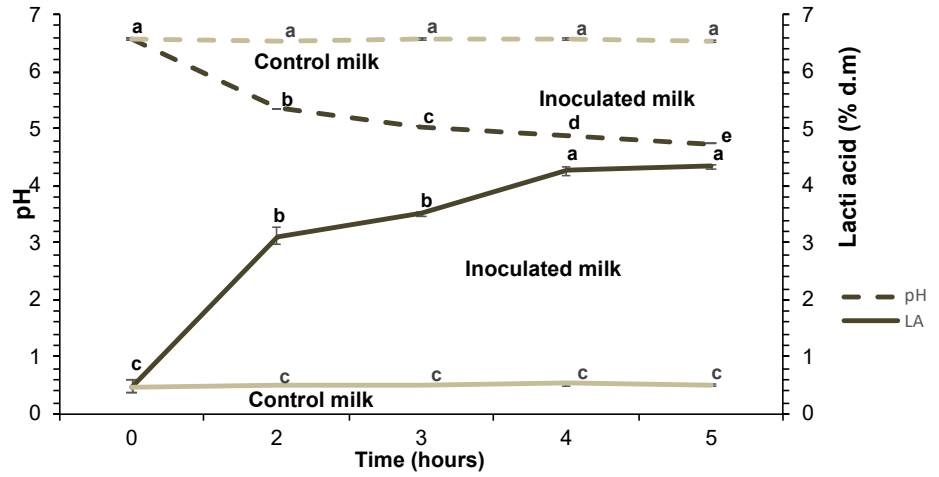
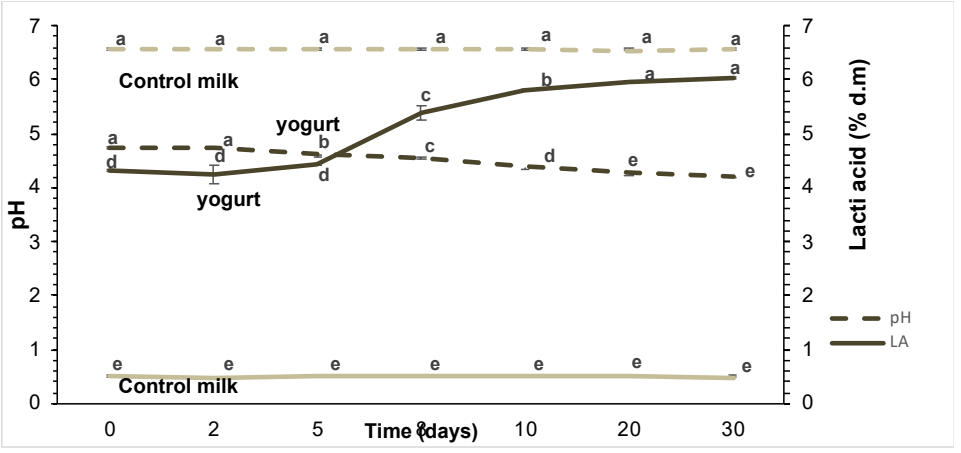


**Figure 2.2** pH and lactic acid (%) during the five-hour incubation at 40 °C in inoculated milk (a) and during storage at 4 °C in yogurt (b) compared to the uninoculated milk (control).

**(a**

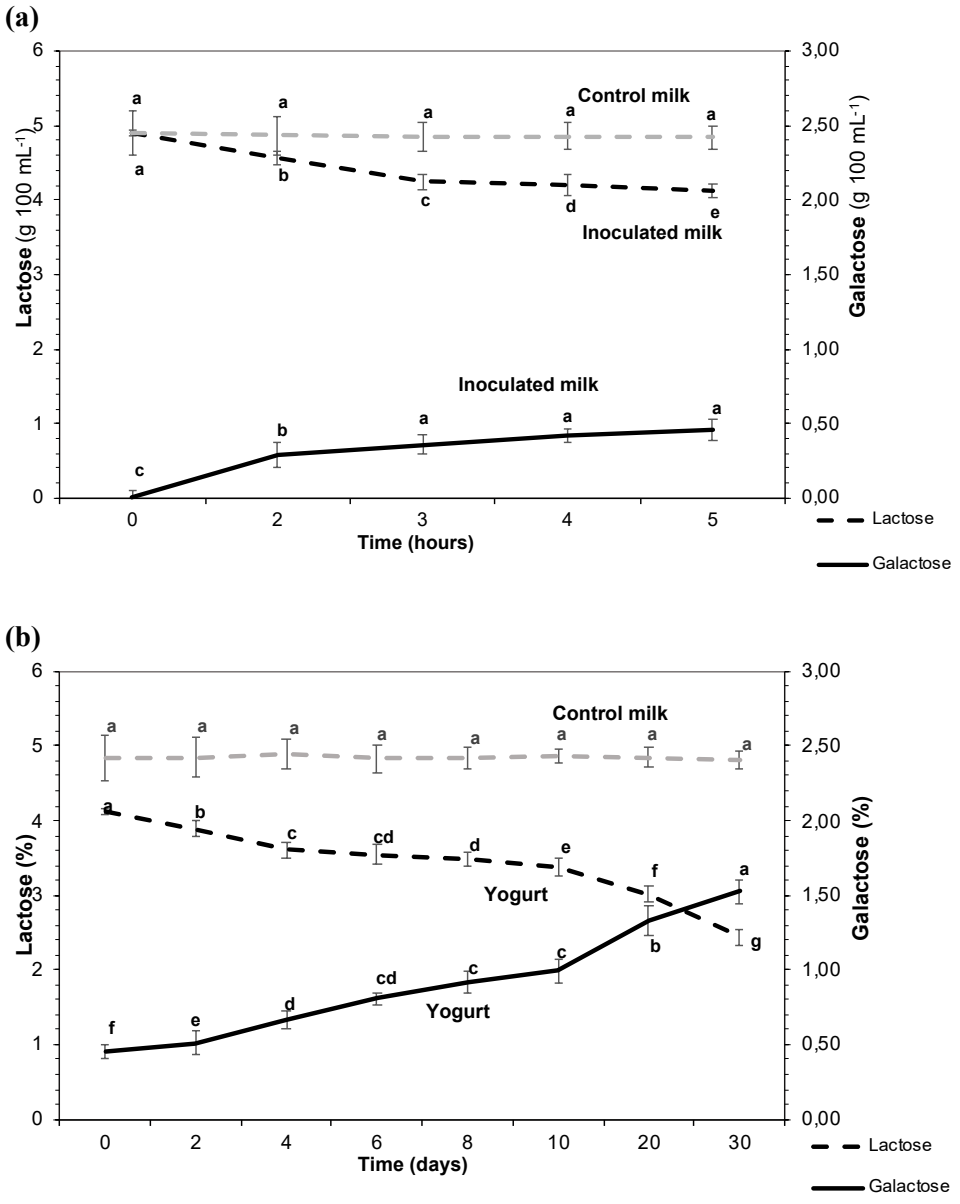



**(b)**




Different letters on the same line indicate statistically significant differences ($p$ value < 0.05).

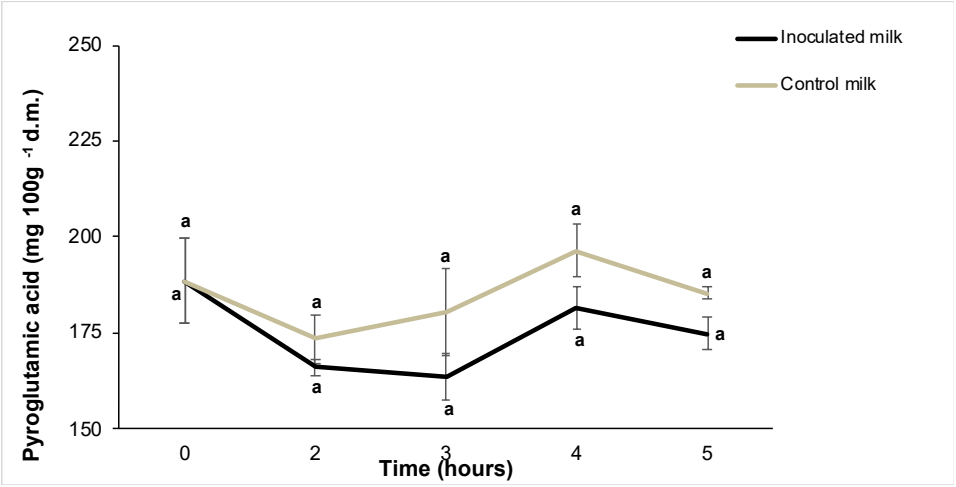
**Figure 2.3** Lactose and galactose (g 100 $mL^{-1}$) during the five-hour incubation at 40 °C in inoculated milk (a) and during storage at 4 °C in yogurt (b) compared to the uninoculated milk (control).



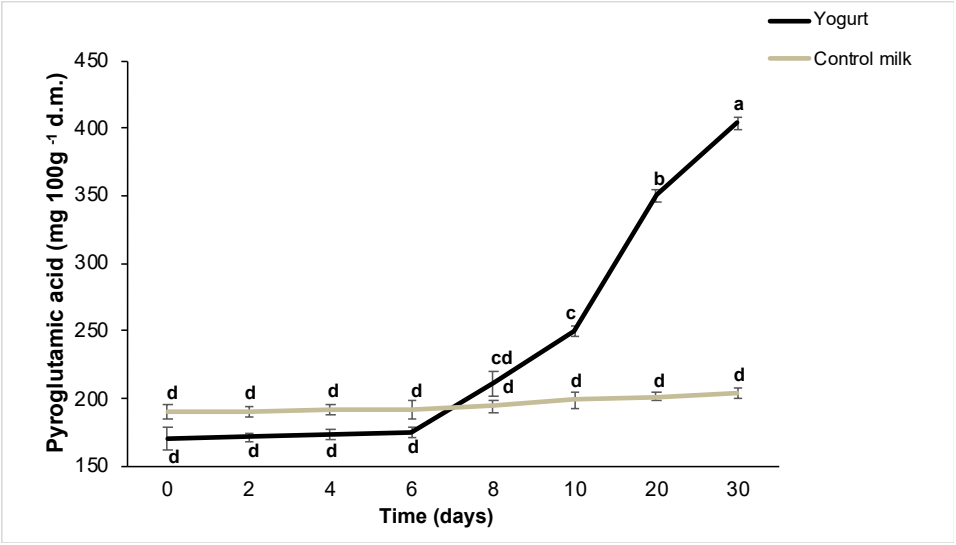

Different letters on the same line indicate statistically significant differences ($p$ value < 0.05).

**Figure 2.4** Kinetics of pyroglutamic acid formation during the five-hour incubation at 40 °C in inoculated milk (a) and during storage at 4 °C in yogurt (b) compared to the uninoculated milk (control).

**(a)**




**(b)**




Different letters on the same line indicate statistically significant differences ($p$ value < 0.05).

# CHAPTER 3. PRODUCTION OF BUTYRIC ACID BY DIFFERENT STRAINS OF *LACTIPLANTIBACILLUS PLANTARUM*

Submitted paper

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# CHAPTER 4. KINETICS OF FORMATION OF BUTYRIC AND PYROGLUTAMIC ACID IN PROBIOTIC, PREBIOTIC AND SYNBIOTIC YOGHURT

Paper in submission

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# CHAPTER 5. PYROGLUTAMIC ACID IN MILK: INFLUENCE OF HEAT TREATMENT AND ENZYMATIC ACTIVITY OF LACTOBACILLI

**ABSTRACT**

In the previous chapters, the presence of pyroglutamic acid in milk has been reported. This study aimed to understand the influence of heat treatment and the enzymatic activity of lactobacilli in the production of this acid. For this reason, different types of drinking milk (from raw to sterilized) and milk inoculated with different strains of Lactobacilli with or without glutamine were analyzed. UHT sterilized milk showed the highest content of pyroglutamic acid (23 mg/100g), confirming a positive correlation between heat treatment temperature and the content of this acid. As for the lactobacilli tested, *Lactobacillus bulgaricus* produces pyroglutamic acid even without glutamine enrichment. On the other hand, the other lactobacilli tested to produce high quantities of pyroglutamic acid only when glutamine is added.

**Keywords:** pidolic acid; heat treatment; fermentation test; *Lactobacillus* spp.;

## 5.1 INTRODUCTION

Pyroglutamic acid is an amino acid found in a variety of processed and unprocessed foods. It is formed following the spontaneous cyclization of glutamic acid and glutamine at high temperatures or by the activity of glutamine cyclase and pyrrolidone carboxyl peptidase activities, released by microbial cultures, such as *Lactobacillus helveticus, Lactobacillus delbrueckii* subsp. *bulgaricus*, *L. delbrueckii* subsp. *lactis* and *Streptococcus thermophilus* (**Mucchetti *et al.*, 2000; Mucchetti *et al.*, 2002**). Previous studies have reported that in various acidic food matrices (grape juice and tomato puree) the presence of pyroglutamic acid can be correlated to the high treatment temperatures applied in specific process phases, including pasteurization and classic autoclave sterilization (**Pfeiffer & König, 2009**). The presence of pyroglutamic acid in some types of milk (human, donkey milk and formula milk) has been reported by **Murgia *et al.* (2019**). However, the impact of heat treatment on the concentration of pyroglutamic acid in the finished product has not been investigated for drinking milk.

Therefore, the purpose of this part of the project is to evaluate the effect of heat treatments on the pyroglutamic content in milk, analyzing raw, pasteurized and sterilized. Furthermore, different strains of homofermentative *Lactobacilli* were tested with and without the addition

of glutamine in milk to evaluate the ability of the single species to produce pyroglutamic acid.

## 5.2 MATERIALS AND METHODS

### 5.2.1 *Materials*

The different samples of whole milk for the analysis (raw, HTST pasteurized, fresh pasteurized, high quality fresh pasteurized, microfiltered pasteurized, sterilized, UHT and powder) and for the development of a laboratory scale model system (fresh milk high quality pasteurized milk and UHT milk) were found at farms and local markets (**Table 5.1**).

### 5.2.2 *pH determination of milk*

The pH value was determined according to the method described by **Martelli & Frattini** (**1985**) which represents the real acidity of the fermented milk as well as the hydrogen ionic concentration of the same. For this analytical measurement, a bench pH meter (Medidor PH BASIC 20 Crison Instruments, Barcelona, Spain) was used.

### 5.2.3 *Pyroglutamic and lactic acid determination*

Organic acid extraction was carried out according to the method described by **Bevilacqua & Califano** (**1989**), with some modifications. Then, pGlu and lactic acid were determined by HPLC according to the method reported by **Marconi *et al.*** (**2007**), with some modifications, as reported in **chapter 2.**

### 5.2.4 *Fermentation test*

The following cultures belonging to the collection of the Microbiology section of the Department of Agricultural Science (Portici, Naples) were used for the fermentation test:

1. *Streptococcus thermophilus* ATCC 19258
2. *Streptococcus thermophilus* 85
3. *Streptococcus thermophilus* 90
4. *Lactobacillus delbrueckii subsp. bulgaricus*
5. *Lacticaseibacillus casei* ATCC393
6. *Streptococcus macedonicus* 96
7. *Streptococcus macedonicus* 97
8. *Lacticaseibacillus casei* Shirota

Cultures were revitalized in MRS broth and inoculated 2% in pasteurized milk and milk enriched with 7mg/ml of glutamine. They were then incubated for 48 h at 37 ° C. A milk sample without inoculation was incubated under the same conditions and used as a control. After incubation, samples were frozen at -18°C until the analysis.

### 5.2.*5 Statistical analysis*

All experiments and determinations were performed in triplicate, and the 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).

## 5.3 RESULTS AND DISCUSSIONS

### *5.3.1 Effect of heat treatment on pGlu content in milk*

Table 5.2 shows that as the treatment temperatures increase, the pH of the milk decreases. This is explained by the fact that there is a simultaneous decrease in the concentration of ionic calcium (**On-Nom *et al*., 2010**). As regards the content of pyroglutamic acid, an increasing trend can be observed with increasing treatment temperature even if not statistically significant. The minimum concentration (14.40 mg/100g) was found in raw milk, which has not undergone any thermal treatment. However, the pre-existence of a natural quantity of pyroglutamic acid in drinking milk not subjected to any type of technological process after milking is probably due to the presence of enzymes of bacterial or endogenous origin of the hydrolytic type (**Corradini,1995**) capable of identifying glutamine as a substrate for the subsequent cyclization in pGlu. UHT sterilized milk instead showed a higher concentration of pGlu (23.28 mg/100 g) higher than the milk subjected to classic sterilization (20.10 mg / 100 g); so, the cyclization phenomenon of the amino acid glutamine in milk, which occurs in thermal conditions ≥ 100°C, could be dependent on the temperature reached during the process and not on the treatment time.

### *5.3.2 pGlu production in milk by different strains of Lactobacilli*

Figure 5.1 shows the content of pyroglutamic acid in milk samples inoculated with different strains of homofermentative lactobacilli. As can be seen, only *L.bulgaricus* was capable of producing pyroglutamic acid in the absence of glutamine. This could be related to a possible aminopeptidase activity, detected in non-starter lactic acid bacteria by **William & Banks (1997**), that allowed the release of pGlu present as a terminal residue in milk proteins. Moreover, this was in line with the results obtained by **Aiello *et al.* (2021)** for the production of traditional yoghurt on a laboratory scale. The traditional yoghurt, produced with *S. thermophilus* and *L. bulgaricus*, had shown a higher content of pyroglutamic acid than uninoculated control milk.

On the other hand, when glutamine was added to milk, the lactobacilli all produced greater quantities of pGlu. *S. thermophilus* ATCC 19258 produced the least amount (91.74mg/kg), while *L.casei* Shirota was the greatest (194.82mg/100g). These results confirmed that the lactobacilli tested possessed enzymes capable of cycling the glutamine present in the medium, as reported by **Mucchetti *et al.* (2000)** and **Mucchetti *et al.*** (**2002**).

## 5.4 CONCLUSIONS

The content of pyroglutamic acid was influenced both by the temperature of the heat treatment to which the milk is subjected and by the enzymatic activity of the lactobacilli. Compared to raw milk, UHT milk showed a slightly higher pGlu content (about 64% more).

As regards lactobacilli, the strain-dependent ability to produce pyroglutamic acid has been confirmed, linked to the presence of glutamine cyclase and aminopeptidase. All strains in glutamine-enriched milk produced 79% (*Lactobacillus delbrueckii* subsp. *bulgaricus*) up to 823% (*Lacticaseibacillus casei* Shirota) more pyroglutamic acid than non-enriched milk.

*L. bulgaricus* was the only one capable of producing pyroglutamic acid without enriching milk with glutamine. This would explain the increase in pyroglutamic acid after fermentation and during the storage of yoghurts produced with these species and analyzed in the previous chapters.

Finally, given the variability due to the microbial strain, further fermentation tests are in progress with other strains of homo- and heterofermentative lactobacilli.

## References


Aiello, A., Pepe, E., De Luca, L., Pizzolongo, F., & Romano, R. (2022). Preliminary study on kinetics of pyroglutamic acid formation in fermented milk. *International Dairy Journal*, 126, 105233.

Bevilacqua, A. E., & Califano, A. N. (1989). Determination of organic acids in dairy products by high performance liquid chromatography. *Journal of Food Science*, 54(4), 1076-1076. https://doi.org/10.1111/j.1365-2621.1989.tb07948.x

Corradini, C. (1995). Chimica e tecnologia del latte. Tecniche nuove.

Marconi, O., Floridi, S., & Montanari, L. (2007). Organic acids profile in tomato juice by HPLC with UV detection. *Journal of food quality*, 30(2), 253 – 266.

Martelli, A., & Frattini, C. (1985). Introduzione all'Analisi degli Alimenti. Corso Editore, Ferrara, 1 – 8, 101 – 117.

Mucchetti, G., Locci, F., Gatti, M., Neviani, E., Addeo, F., Dossena, A., & Marchelli, R. (2000). Pyroglutamic acid in cheese: presence, origin, and correlation with ripening time of Grana Padano cheese. *Journal of Dairy Science*, 83(4), 659 – 665.

Mucchetti, G., Locci, F., Massara, P., Vitale, R., & Neviani, E. (2002). Production of pyroglutamic acid by thermophilic lactic acid bacteria in hard-cooked mini- cheeses. *Journal of Dairy Science*, 85(10), 2489 – 2496.

Murgia, A., Scano, P., Cacciabue, R., Dessì, D., & Caboni, P. (2019). GC-MS metabolomics comparison of yoghurts from sheep's and goats' milk. *International Dairy Journal*, 96, 44 – 49.

On-Nom, N., Grandison, A. S., & Lewis, M. J. (2010). Measurement of ionic calcium, pH, and soluble divalent cations in milk at high temperature. *Journal of dairy science*, 93(2), 515 – 523.

Pfeiffer, P., & König, H. (2009). Pyroglutamic Acid: A Novel Compound in Wines. In: König H., Unden G., Fröhlich J. (eds) Biology of Microorganisms on Grapes, in Must and in Wine, Springer, Berlin, Heidelberg.

Williams, A. G., & Banks, J. M. (1997). Proteolytic and other hydrolytic enzyme activities in non-starter lactic acid bacteria (NSLAB) isolated

from Cheddar cheese manufactured in the United Kingdom. *International Dairy Journal*, 7(12), 763-774.

**Table 5.1** Samples of drinking milk

| Type of drinking milk | Treatment thermal | Internal code |
|---|---|---|
| Raw milk | - | LC |
| High quality pasteurized fresh milk | 72-75 ° C x 15 " | DAQ |
| Microfiltered pasteurized milk | 72-75 ° C x 15 " | LMP |
| Fresh pasteurized milk | 72-75 ° C x 15 " | LPF |
| HTST pasteurized milk | 72-75 ° C x 15 " | HTST |
| Sterilized milk | 120 ° C x 15-20 ' | LS |
| UHT sterilized milk | 135 ° C x 1 " | UHT |

**Table 5.2** Determination of pH and pyroglutamic acid, expressed in mg/100 g of product, in milk subjected to different types of heat treatment. The results are expressed as mean ± ds and different letters in the same column indicate statistically significant differences ($p < 0.05$).

| Type of drinking milk | pH | pGlu (mg/100g) |
|---|---|---|
| **LC** | $6.69 \pm 0.01^{a}$ | $14.40 \pm 0.65^{c}$ |
| **DAQ** | $6.54 \pm 0.02^{b}$ | $17.10 \pm 0.37^{bc}$ |
| **LMP** | $6.60 \pm 0.01^{b}$ | $17.71 \pm 0.96^{bc}$ |
| **LPF** | $6.61 \pm 0.01^{b}$ | $18.13 \pm 0.87^{bc}$ |
| **HTST** | $6.70 \pm 0.01^{a}$ | $19.73 \pm 0.89^{bc}$ |
| **LS** | $6.39 \pm 0.01^{b}$ | $20.10 \pm 0.63^{bc}$ |
| **UHT** | $6.58 \pm 0.00^{c}$ | $23.28 \pm 1.01^{b}$ |

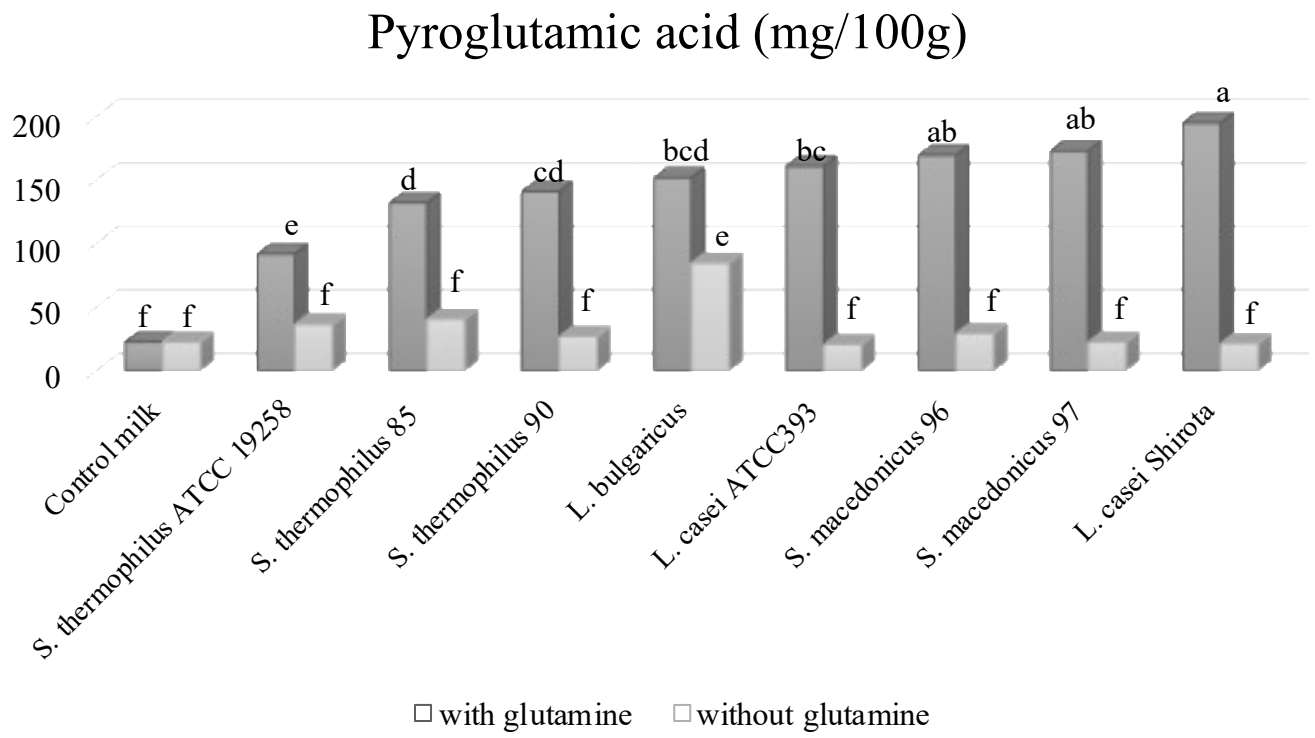


**Figure 5.1** Pyroglutamic acid in milk with and without glutamine inoculated with different strains of *Lactobacilli*.

# CHAPTER 6. SUMMARY, CONCLUSIONS AND FUTURE PERSPECTIVES

Butyric acid and pyroglutamic acid are two small molecules with great properties, especially as regards the (antitumoral, mitogenic, anxiolytic, antidiabetic, hypolipidemic, and anti-inflammatory) effects on human health.

In **chapter 1** the details on these beneficial effects are reported to better understand the importance from the nutritional point of view of these molecules. Furthermore, the currently known formation mechanisms are reported and for butyric acid, there is no clear information about the production by lactobacilli. As for pyroglutamic acid, it has been found in many food matrices but to the best of our knowledge, there are no data in literature about its content in fermented milk.

**Chapter 2** thus focuses on the content of pyroglutamic acid in different types of fermented milk (yogurt, kefir, etc.) already on the market, and in a traditional yoghurt made on a laboratory scale. Yoghurt, kefir and other probiotic fermented milk contained it ranging from 51.65 to 277.37mg/100g d.m. as a function of the thermophilic lactic acid bacteria used as starter cultures. In the yoghurt production process, pyroglutamic acid was constant during the fermentation phase and increased during the storage phase. **Chapter 3** focuses, instead, on butyric acid and in particular its production by some strains of *Lactoplantibacillus plantarum,* inoculated in different growth media. In particular, it was found that these strains

surprisingly produced butyric acid only when there was a lipid and non-oligosaccharide source in the medium.

Subsequently, in **chapter 4** the production of butyric and pyroglutamic acid in prebiotic, probiotic and symbiotic yoghurt was also studied. Probiotic yoghurt presented the highest content of pGlu (136.6mg/100g), while the lowest content was present in milk (113.5mg/100g). At 10 days of storage an increase in pGlu was visible in all samples due to the gradual conversion of glutamine and to the possible hydrolysis of pGlu from the terminal ends of the proteins by fermenting microorganisms. As for free butyric acid, conventional yoghurt exhibited a higher concentration (2.13-2.36mg/100g) than control milk (1.77-1.81mg/100g). In fact, during fermentation, small quantities of free fatty acids were released due to the activity of lipases and microbial esterases. Prebiotic yoghurt showed concentrations of butyric not different from conventional yoghurt. Fiber may not affect the metabolic activity of the lactobacilli, as has been shown by the fermentation tests on *L.plantarum*. In contrast, probiotic yoghurt showed a higher concentration of butyric acid (2.03-2.76mg/100g) than conventional yoghurt. Synbiotic yoghurt with 1% and 3% prebiotics exhibited the highest concentrations of butyric acid: 2.72-2.97 and 2.56-2.81mg/100g, respectively. This could be due to the presence of oligosaccharides, which could ensure a better survival of *L. acidophilus* and *B. bifidum* in yoghurt.

In **Chapter 5** the pGlu content already present in the control milk used for the previous experiments was explained. It was found to be linked to heat treatment, although it is also present in raw milk. Furthermore, fermentation tests in milk with glutamine supplements were conducted to individually evaluate the production of pGlu by various lactic bacteria. In this regard, among the lactobacilli tested, *L.bulgaricus* was able to produce pyroglutamic in milk even without the addition of glutamine used as a precursor while the others were not.

In conclusion, this study allowed us to:

- generate a database that is currently absent in the literature about the pyroglutamic content in different types of fermented milk already available on market. In particular, the tested samples of yoghurt, kefir and other probiotic fermented milk contained pGlu as a function of the thermophilic lactic acid bacteria used as starter cultures.
- Understand that the butyrogenic capability of all *L. plantarum* strains tested was related to lipase-mediated triglyceride hydrolysis and not to oligosaccharides fermentation. The false attribution of the production of SCFAs (especially of butyric acid) as a result of oligosaccharides fermentation was demonstrated by the use of the synthetic fat-free medium where butyric acid was no longer

produced at all. Accordingly, in the media supplemented with tributyrin the release of butyric acid was significantly higher and attributed to lipase-mediated triglyceride hydrolysis associated with l*actobacillus* strains.

- Evaluate the positive influence of the addition of probiotic strains to the traditional starter culture on the synthesis of pyroglutamic acid and butyric acid, which was also intensified by the addition of prebiotics in the yoghurt, resulting in favourable growth conditions.
- conclude that the presence of pyroglutamic acid (188.47 mg 100 $g^{-1}$ d.m.) in the milk used to produce yoghurt at the beginning of fermentation, was slightly dependent on the thermal treatment because is already present in raw milk. Furthermore, each strain of lactobacilli tested produced pyroglutamic acid in milk only when there was enrichment in glutamine. *Lb. bulgaricus* produced pyroglutamic acid also without the addition of glutamine in milk.

These are interesting results, especially in light of the several beneficial effects on human health and the possibility of using lactobacilli to produce these two molecules. However, further *in vitro* and *in vivo* studies are necessary to demonstrate the bioaccessibility and bioavailability of pGlu in milk and fermented milk.