

Fabrizio Domenico Nicosia

**DEVELOPMENT OF A PLANT-BASED MILK-CLOTTING ENZYME AND A
DEBITTERING MICROBIAL CULTURE FOR THE FORMULATION OF HALAL-
CERTIFIED HEALTHY CHEESE**

Thesis submitted to the Food Science and
Technology Graduate Program of the
Universidade Federal de Viçosa in partial
fulfillment of the requirements for the degree of
Doctor Scientiae.

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Abstract

Nicosia, Fabrizio Domenico, D.Sc., Universidade Federal de Viçosa, April, 2024. **Development of a plant-based milk-clotting enzyme and a debittering microbial culture for the formulation of halal-certified healthy cheese.** Adviser: Antônio Fernandes de Carvalho and Co-advisers: Cinzia Lucia Randazzo and Cinzia Caggia.

In recent years, there has been a significant shift in consumer preferences towards healthier and more diversified food options, with a particular emphasis on products derived from vegetable sources. This evolving trend is driven by an increased awareness of the link between diet and well-being, as well as a growing desire for sustainable and ethically sourced food choices. Consumers are becoming more health-conscious, with a heightened awareness of the impact of their dietary choices on overall well-being. The emphasis on plant-based diets, rich in fruits and vegetables, is supported by numerous studies linking diets to reduced risks of chronic diseases, to improved cardiovascular health, and to enhanced longevity. The modern consumer is increasingly seeking variety and novelty in their food choices, moving beyond traditional products. Vegetable-based products offer a diverse array of flavors, textures, and culinary experiences, appealing to consumers looking to experiment with their senses. The availability of innovative plant-based alternatives to traditional animal products, such as plant-based substitutes, has resulted in significant changes also in the dairy industry. In detail, the dairy market needs the search for animal rennet substitutes, because of the growth of vegetarian's market niche as well as the request for Kosher and Halal foods. The role of milk-clotting enzymes is crucial in cheesemaking; among them, animal rennet is the most ancient milk-clotting enzyme and still the most widely used biocatalyst in cheesemaking procedures. The clotting properties of animal rennet are due to chymosin, an aspartic protease extracted from the abomasum of new-born ruminants with high specificity for cleaving k-casein Phe₁₀₅-Met₁₀₆ bond. However, the changed perception of the consumer, coupled to a diminishing supply of animal rennet, are responsible of the high demand for alternative milk-clotting enzymes. Several new vegetable sources of milk clotting enzymes have been investigated in the last twenty years and the development of new plants derived milk-clotting enzymes is now in progress. Plant enzymes are generally extracted by aqueous maceration of different plant organs, such as flowers, seeds, roots or leaves, followed by several different homogenization procedures. One innovative milk-clotting enzyme is Actinidin (EC 3.4.22.14), a cysteine protease extracted from kiwifruit (*Actinidia deliciosa*), that represents an interesting alternative to chymosin for milk coagulation. Previous studies demonstrated that the actinidin forms milk clots under the typical conditions used in cheese manufacturing. Nevertheless, plant proteases are characterized by a high proteolytic activity that generates bitter flavours in the final product. To reduce the bitter taste caused by the strong proteolytic activity, strains of Lactic Acid Bacteria (LAB) with high aminopeptidase activity are promising. Specific aminopeptidases (like Pep X and Pep N) are able to hydrolyze the bitter peptides formed during cheese ripening.

Keywords: Milk-clotting enzymes, cheesemaking, vegetable coagulant, actinidin, lactic acid bacteria, aminopeptidase activity.

RESUMO

Nicosia, Fabrizio Domenico, D.Sc., Universidade Federal de Viçosa, abril de 2024. **Development of a plant-based milk-clotting enzyme and a debittering microbial culture for the formulation of halal-certified healthy cheese.** Orientador: Antônio Fernandes de Carvalho e Co-orientadores: Cinzia Lucia Randazzo e Cinzia Caggia.

Nos últimos anos, a preferência dos consumidores por alimentos mais saudáveis e diversificados à base de produtos de origem vegetal apresentou uma mudança significativa. Essa tendência é impulsionada por uma maior conscientização sobre a relação entre dieta e bem-estar, bem como por um crescente desejo por escolhas alimentares sustentáveis e éticas. Os consumidores estão se tornando mais conscientes em relação à saúde, com uma maior preocupação com o impacto de suas escolhas alimentares no bem-estar geral. A ênfase em dietas baseadas em plantas, ricas em frutas e vegetais, é apoiada por inúmeros estudos que relacionam as dietas a redução dos riscos de doenças crônicas, melhoria da saúde cardiovascular e aumento da longevidade. O consumidor moderno está cada vez mais buscando variedade e novidade em suas escolhas alimentares, indo além dos produtos tradicionais. Os produtos à base de vegetais oferecem uma ampla variedade de sabores, texturas e experiências culinárias, atraindo consumidores que desejam testar seus sentidos. O surgimento de alternativas inovadoras à base de plantas para produtos animais tradicionais, também resultou em mudanças significativas na indústria de laticínios. Em detalhes, o mercado de lácteos tem visto a necessidade de buscar substitutos para coalho animal, devido ao crescimento do nicho de mercado vegetariano, bem como à demanda por alimentos kosher e halal. O papel das enzimas de coagulação do leite é crucial na fabricação de queijos. Entre elas, o coalho animal é a enzima de coagulação do leite mais antiga e ainda a mais utilizada na fabricação de queijos. As propriedades coagulantes do coalho animal são devidas à quimosina, uma protease aspártica extraída do abomaso de ruminantes recém-nascidos com alta especificidade para clivar a ligação Fhe105-Met106 da κ -caseína. No entanto, a mudança na percepção do consumidor, juntamente com a redução no fornecimento de coalho animal, são responsáveis pela alta demanda por enzimas alternativas de coagulação do leite. Várias enzimas coagulantes de fontes vegetais têm sido investigadas nos últimos vinte anos e o desenvolvimento de novas enzimas capazes de coagular o leite derivadas de plantas está em progresso. As enzimas vegetais geralmente são extraídas por maceração aquosa de diferentes órgãos vegetais, como flores, sementes, raízes ou folhas, seguida por vários procedimentos de homogeneização diferentes. Uma enzima de coagulação do leite inovadora é a Actinidina (EC 3.4.22.14), uma protease de cisteína extraída do kiwi (*Actinidia deliciosa*), que representa uma alternativa interessante para a substituição da quimosina. Estudos anteriores demonstraram que a actinidina forma coágulos de leite nas condições típicas usadas na fabricação de queijos. No entanto, as proteases vegetais são caracterizadas por uma alta atividade proteolítica capaz de gerar sabores amargos no produto final. Para reduzir o sabor amargo causado pela forte atividade proteolítica, cepas de bactérias do ácido láctico (BAL) com alta atividade de aminopeptidase são promissoras. Aminopeptidases específicas (como Pep X e Pep N) são capazes de hidrolisar os peptídeos amargos formados durante o amadurecimento do queijo.

Palavras-chave: Enzimas coagulantes do leite, fabricação de queijo, coagulante vegetal, actinidina, bactérias do ácido láctico, atividade aminopeptidase,

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1. Research highlights

- Actinidin, the milk-clotting enzyme present in kiwifruit (*Actinidia deliciosa*) is a promising candidate for the formulation of a new plant coagulant.
- Temperature of 40°C in combination with a pH value of 5.5 are the optimum parameters for actinidin enzyme extract coagulant.
- A rapid, cost-effective, chemical-free, and environmentally friendly approach, based on kiwifruit aqueous extract, was developed.
- Selected LAB strains with high aminopeptidase activity were screened for the hydrolyzation of bitter peptides.
- *Lactococcus lactis* subsp. *lactis* bv. *diacetylactis* Q5C6 strain exhibited the highest Pep X and Pep N activities.
- *L. lactis* subsp. *lactis* bv. *diacetylactis* Q5C6 strain was used as adjunct culture in experimental cheesemaking to reduce the bitter taste caused by kiwifruit enzymatic extract.
- The cheeses made with the kiwifruit enzymatic extract showed a higher polyphenol content than those clotted with commercial coagulants.

2. Outline of the thesis

This thesis deals with the development of a new plant coagulant and of an adjunct microbial culture for the formulation of a Halal-certified cheese. In detail, the objective of this study was to: (i) develop a vegetable coagulant extracted from kiwifruit in order to set up a substitute to animal rennet coagulant for Halal cheesemaking; (ii) select an adjunct culture composed by Lactic Acid Bacteria strains in order to reduce the bitter aftertaste of the cheese, generated by the high proteolytic activity of the plant extract; (iii) set up an experimental cheesemaking by using a combination of plant extract with a selected adjunct culture. The thesis consists of the following chapters:

Chapter 1: is an extensive literature review that deals with all types of vegetable coagulants used in cheesemaking. The reduced availability and increasing prices of calf rennet, along with the rising global demand for cheese, have prompted worldwide exploration of alternative clotting enzymes to replace traditional rennet in cheese production. Furthermore, factors such as religious considerations and the preferences of vegetarian consumers have driven the search for alternative rennet substitutes. Nowadays, there are various plant-derived milk-clotting enzymes available for cheesemaking. Extensive efforts have been made to compare their effects on the texture and taste of cheese in comparison to animal rennet. Nevertheless, vegetable milk-clotting enzymes still face challenges in the cheesemaking process, primarily due to their excessive proteolytic activity, which can contribute to the development of a bitter flavor to the cheese. This review offers an overview of the most utilized vegetable milk-clotting enzymes in cheese technology, categorized by their protease class. In detail, the milk-clotting and proteolytic activities of these enzymes are discussed in relation to their application in different cheese products. Finally, the strengths and weaknesses of coagulants and the possible strategies to implement their use at industrial scale are evaluated.

This work presented has been published in Foods:

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Chapter 2: is a research article, which describes the development and the use of a kiwifruit aqueous extract as a milk-clotting enzyme for cheesemaking. In detail, polyacrylamide gel electrophoresis (SDS-PAGE) was employed to evaluate the presence of actinidin, the kiwifruit enzyme responsible for κ -casein hydrolysis, in the pulp and in the peel of ripe and unripe kiwifruits. Data revealed the presence of the enzyme in both the peel and the pulp of the fruit. However, the aqueous extract obtained from the pulp exhibited hydrolytic activity towards both κ -casein and semi-skimmed milk. To optimize the milk-clotting activity of the kiwifruit pulp extract, various temperature and pH conditions were evaluated to achieve the highest MCA/PA (Milk-Clotting Activity/Proteolytic Activity) ratio. The best performance was obtained at a temperature of 40 °C and a pH value of 5.5. Additionally, data indicated a greater hydrolytic activity in the enzymatic preparation from ripe kiwifruits compared to unripe ones, suggesting the use of pulp extract from ripe kiwifruits in laboratory-scale cheesemaking. Notably, results showed that the inoculum of 3% (v/v) of the ripe kiwifruit pulp extract resulted in a curd yield of 20.27%, which was comparable to the yield achieved with chymosin. Hence, the extraction method for kiwifruit aqueous extract presented in this study proved to be a rapid, cost-effective, chemical-free, and environmentally friendly approach for utilizing plant-derived coagulants in cheese manufacturing.

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Chapter 3: is a research article focuses on the selection of lactic acid bacteria strains with interesting technological characteristics for cheesemaking. Special attention was paid to the evaluation of the aminopeptidase activity (Pep N and Pep X) capable of reducing the bitter taste given by vegetable coagulants. A total of 26 lactic acid bacteria isolates obtained from Italian and Brazilian cheeses were subjected to several tests to assess their suitability in cheesemaking. These isolates were evaluated for their salt tolerance, exopolysaccharide and diacetyl production, lipolytic, acidifying, and proteolytic activities. Additionally, their aminopeptidase (Pep N and Pep X) activities were examined. Most of the strains displayed salt tolerance up to 6% NaCl, with only two *L. delbrueckii* strains (P14, P38), one *L. rhamnosus* strain (P50), and one *L. plantarum* strain (Q3C4) showing the ability to grow in the presence of 10% NaCl. All strains, except 2 *L. plantarum* strains (Q1C6 and Q3C4), exhibited either low or moderate acidifying activity and good proteolytic characteristics. In addition, the production of exopolysaccharides (EPS) and diacetyl was observed among the tested strains. None of them exhibited lipolytic activity. Regarding aminopeptidase activities, *L. delbrueckii* P10 strain, *L. rhamnosus* P50, strain and *L. lactis* Q5C6 strain demonstrated high debittering activity displaying high values of both Pep N and Pep X. Based on the findings presented here, the strains *L. delbrueckii* P10, *L. rhamnosus* P50, and *L. lactis* (Q5C6), could be considered as promising adjunct cultures for cheesemaking process.

This work presented in Chapter 3 has been published in Foods:

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Chapter 4: deals with the use of *Lactococcus lactis* subsp. *lactis* bv. diacetylactis Q5C6 strain in experimental cheesemaking as a debittering adjunct culture. Firstly, the study aimed at defining the optimal quantity of kiwifruit enzymatic extract for laboratory-scale coagulation tests. Then, two distinct cheesemaking trials (A and B) were conducted, and the resulting cheeses were evaluated through physico-chemical, microbiological, and sensory analysis. Results showed that, the cheeses made with the kiwifruit enzymatic extract exhibited different fat, ash, and protein content respect to ones clotted by animal rennet. The *L. lactis* subsp. *lactis* bv. diacetylactis Q5C6 strain, inoculated at 1%, as adjunct culture, was able to reduce the bitter taste, improving the sensory profile of the final product.

This work presented in Chapter 4 has been published in Food Bioscience:

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Chapter 5: In the present study, cheeses coagulated with different milk-clotting enzymes were investigated. In detail, commercial animal rennet, microbial coagulant, and kiwifruit enzymatic extract were used in the cheesemaking process, and the different cheeses were examined. In detail, microbiological and chemical analysis were carried out and the organoleptic profiles were assessed analytically through E-Eye, E-Nose and E-Tongue platforms. Results obtained from the microbiological analysis showed higher presence of lactobacilli and lactococci in cheeses clotted with animal rennet and vegetable coagulant. This data is correlated to the higher presence in these samples of carboxylic acids, aldehydes, alcohols, and other compounds derived from the metabolism of the LAB, that are important components with a key role in the flavor development of cheese. These data were confirmed by artificial senses analysis, showing that cheese coagulated with vegetable enzyme exhibited organoleptic profile comparable to the cheese coagulated with traditional animal rennet. Additionally, the vegetable clotted cheese revealed higher phytosterols and polyphenols compounds as well as a lower presence of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) allowing to obtain a final product with healthy and safety properties.

This work presented in Chapter 5 will be submitted to Food Bioscience journal.

Other activities: is as a collection of research articles, projects and conference participations carried

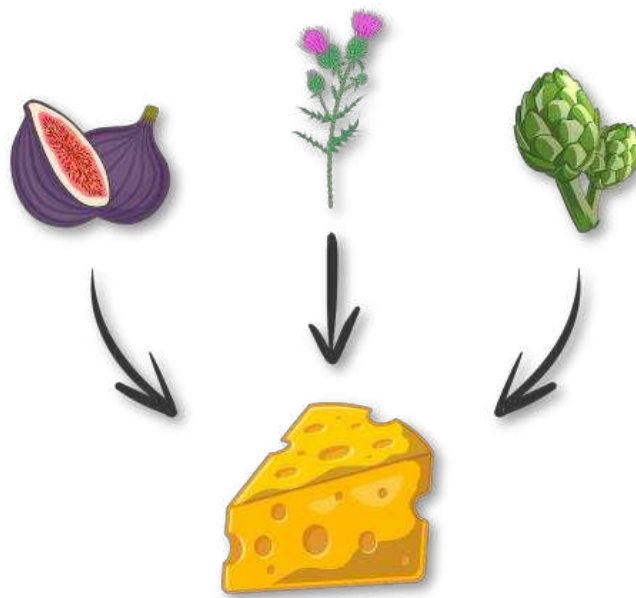
out during the PhD cycle.

The research article “Formulation of germinated brown rice fermented products functionalized by probiotics” aimed to formulate fermented brown rice products with enhanced nutritional and health-promoting properties. Germinated brown rice is considered a functional food in relation to the presence of beneficial nutrients and bioactive compounds in considerable amounts. The present study, evaluated, for the first time, the suitability of germinated brown rice for the production of value-added fermented products functionalized by the addition of probiotics. Four different fermented products with (EYGG, EYBB536, EYBB12, and EYM) and without (EY) probiotics addition were formulated. Microbiological and chemical profiles, presence of bioactive compounds, antioxidant activity, sensory attributes, and shelf-life during refrigerated storage were evaluated. Results showed that the fermentation process determined the improvement of both bioactive compound profile and anti-inflammatory properties of germinated brown rice. All the fermented products were microbiologically stable during refrigerated storage at +4 °C for 28 days. In addition, a high count of both lactobacilli and bifidobacteria was achieved during the shelf-life, indicating the suitability of the germinated brown rice as a probiotic carrier. Based on the sensory profile, high acceptability scores were attributed by panelists to the germinated brown rice experimental products. Based on the aforementioned results, germinated brown rice can be processed as a new fermented formulation with potential health benefits.

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Chapter 1: Review article



1. Chapter 1 - Review article: Plant Milk-Clotting Enzymes for Cheesemaking

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1.1 Abstract

The reduced availability and the increasing prices of calf rennet, coupled to the growing global demand of cheese has led, worldwide, to explore alternative clotting enzymes, capable to replace traditional rennet, during the cheesemaking. In addition, religious factors and others related to the vegetarianism of some consumers, have led to alternative rennet substitutes. Nowadays, several plant-derived milk-clotting enzymes are available for cheesemaking technology. Many efforts have also been made to compare their effects on rheological and sensory properties of cheese to those arising from animal rennet. However, vegetable clotting enzymes are still partially suitable for cheesemaking, due to excessive proteolytic activity, which contribute to bitter flavor development. This review provides a literature overview of the most used vegetable clotting enzymes in cheese technology, classified according to their protease class. Finally, clotting and proteolytic activities are discussed in relation to their application on the different cheesemaking products.

1.2 Introduction

Cheesemaking is a dynamic process in which different technological steps, such as heat treatment, homogenization, and milk coagulation can affect the structure of the milk and determine the characteristics of the final product. Milk coagulation is a crucial step in cheesemaking and the choice of the specific clotting enzyme is fundamental for cheese yield, texture, and flavor. The widely used milk-clotting enzyme is chymosin (EC 3.4.23.4), an aspartic protease, which can hydrolyze a specific peptide bond (Phe₁₀₅-Met₁₀₆) present in κ -casein (Kumar et al. 2010). The C-terminal region of κ -casein is typically hydrophilic and has a negative charge at the native pH of milk (pH 6.7). It covers the casein micelles forming a layer that provides the stability of the micelles through steric hindrance and electrostatic repulsion, once hydrolyzed, the first phase of renneting begins (Britten and Giroux 2022). Casein micelle core is formed by submicells of α - and β -caseins linked together by hydrophobic interactions and by insoluble calcium phosphate, which represents a cross-linking agent (Lee et al. 2009). During cheesemaking, the reduction in milk pH by starter cultures leads to the solubilization of a part of the calcium phosphate, which is released as Ca²⁺. The latter directly influences the second phase of renneting, further reducing the colloidal stability of casein micelles and leading the aggregation of the micelles in the forming curd (Choi et al. 2007; Lucey et al. 1993; Ozcan et al. 2012). To predict the clot formation and the characteristic flavors and body-texture of the final product, the milk-clotting activity (MCA) and proteolytic activity (PA) ratio should be evaluated. MCA refers to the specificity of hydrolysis of the clotting enzyme towards κ -casein, whereas PA refers to the hydrolysis of proteins present in the curd (mainly consisting of α s1-, α s2-, β -, and κ -casein), which can lead

to the formation of aftertaste in the long term. A ratio similar to chymosin (that is considered as a reference) corresponds to a high-quality coagulant, reflecting a high curd yield and low cheese defects, such as bitter flavors and an excellent final product with desirable firmness (Nitu et al. 2021). Figure 1 illustrates the mechanism of the milk-clotting enzyme process that generates bitter flavor.

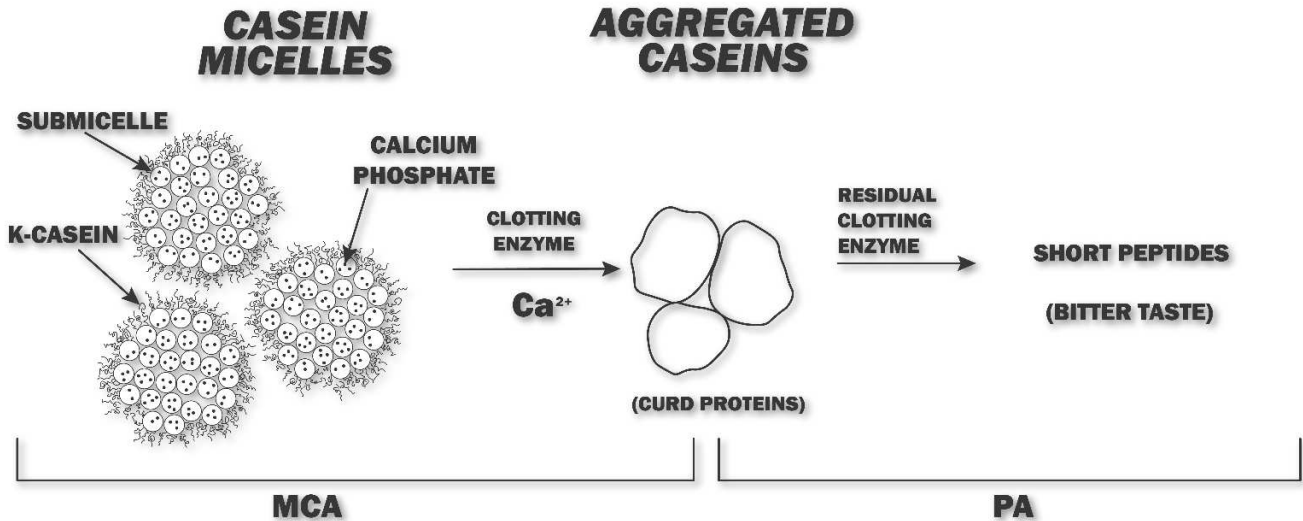


Figure 1. Degradation of caseins by clotting enzyme and development of bitter peptides. MCA= Milk-clotting activity, PA= Proteolytic activity.

Traditionally, chymosin extracted from the abomasum of calves has been used for cheesemaking. However, the reduced availability of calf rennet coupled to the increasing price and growing demand for cheese, have led to search for rennet substitutes coagulants. In fact, nowadays, calf rennet only covers 20–30% of the world demand for milk coagulants (Jacob et al. 2011). In addition, the market is increasingly directed towards diversification of supply to ensure consumers a wide choice, so cheese industries search for alternative coagulants to satisfy consumers who for religious (Islam, Judaism) and ethical reasons (vegetarians) prefer not to consume cheese made with animal rennet. Among the most commercially used substitutes for animal rennet, there are microbial coagulants: aspartic proteases produced by *Rhizomucor miehei* and *Rhizomucor pusillus*, which have a three-dimensional structure similar to chymosin, capable of hydrolyzing, in the same way, the Phe₁₀₅-Met₁₀₆ bond of κ -casein (Aktayeva et al. 2018). The aspartic proteases produced by these filamentous fungi are called mucorpepsin (EC 3.4.23.23) and are synthesized as a precursor: the protease obtained from *R. pusillus* consists of 437 amino acids, 22 of which are signal peptides; the following 44 amino acids are propeptide, and 361 amino acids constitute a mature protease. The precursor of the protease from *R. miehei* comprises a signal peptide of 22 amino acids, a propeptide comprising of 47 amino acids, and a mature protease made up of 361 amino acids (Feijoo-Siota et al. 2014). Another aspartic protease that originates from a microorganism is the endothia pepsin (EC 3.4.23.22) produced by *Cryphonectria parastica*. The precursor consists of 419 amino acids, the signal peptide consists of 20 amino acids, the propeptide of 69 amino acids, and the mature enzyme includes 330 amino acids. Unlike the proteases produced by *Rhizomucor*, this

enzyme hydrolyzes a different site of the κ -casein, the Ser₁₀₄-Phe₁₀₅ bond (Aktayeva et al. 2018). Microbial coagulants have several advantages: low cost of production, and conformity with kosher, halal, and vegetarian eating principles. Their main disadvantages are low specificity, high thermal stability, lower MCA/PA ratio than calf rennet, and more chances of bitterness in resultant cheese (Lebedev et al. 2016). It is advisable that the proteases have low thermal stability, in this way, by regulating the post-cheesemaking temperatures, the residual enzyme survival is reduced, avoiding unwanted proteolysis and, therefore, the development of defects (He et al. 2011). However, the cheeses obtained using endothia pepsin, thanks to its high thermolability, were evaluated as equivalent or even superior in quality than control cheeses produced using animal chymosin (Claverie-Martin and Vega-Hernández 2007). One of the most innovative methods to solve the lack of animal rennet is represented by the Fermentation Produced Chymosin (FPC) or genetic chymosin, which is obtained from a host microorganism such as *Escherichia coli*, *Bacillus subtilis*, and *Lactococcus lactis*, in which the gene for the protease is expressed (Flamm 1991). Cheese is the first food product made using the recombinant DNA technique recognized by the Food and Drug Administration (FDA) (Flamm 1991). Through a reverse transcriptase process, the mRNA of chymosin from the animal abomasum is transferred to cDNA and subsequently grafted into the DNA of a Generally recognized as safe (GRAS) microorganism that will be able to produce chymosin through fermentation (Jacob et al. 2011). The products of fermentation contain chymosin identical to the animal source, meaning that they have the same amino acid sequence as chymosin from the corresponding animal stomach (Harboe et al. 2010). One of the major advantages of this process is that 100% pure chymosin can be obtained, whereas calf rennet is composed of about 80% chymosin and 20% pepsin (that is less specific in hydrolyzing

caseins) (Kumar et al. 2010). One problem is the stringent regulations of some countries towards genetically engineered foods; in fact, FPC is banned in Germany, Netherlands, and France (Egito et al. 2007). The milk-clotting enzyme market is mostly occupied by FPC and, according to statistics, the cheese produced through FPC in the United States and the United Kingdom comprises 70% and 90% of the total cheese production, respectively (Liu et al. 2021).

Alongside the microbial and recombinant milk-clotting enzymes, several animal source coagulants are available on the market and recently have been discussed by Liu and co-workers (Liu et al. 2021). These animal coagulants are interesting rennet substitutes and are suitable for cheese production, generating functional peptides during cheese ripening and flavors compounds appreciated by consumers. However, greater clarity should be paid on cleavage sites and functional peptides after genetic modification (Liu et al. 2021).

1.3 Types and Characteristics of Vegetable Proteases

Recently great attention has increasingly shifted to coagulants of vegetable origin such as proteases present in various plant tissues such as cardoon flower (Zikiou et al. 2020), *Cynara scolymus*' artichoke (Bueno-Gavilá et al. 2020), and *Citrus aurantium* flowers (Mazorra-Manzano et al. 2013). These proteases are enzymes found in plant tissues and can hydrolyze milk caseins at different pH and temperature (Shah et al. 2014).

The use of vegetable coagulants in cheesemaking has very ancient origins. Homer already wrote in the Iliad that fig juice was able to curdle milk. Both Hippocrates in the 5th century BC and Aristotle in the 4th century BC wrote about the use of fig latex to coagulate milk, while Lucius Junius Moderatus Columella in his treatise on agriculture, *De Re Rustica*, in the 1st century BC, mentions

for the first time the use as a coagulant of wild thistle flowers, of seeds of *Carthamus tinctorius* and thyme, claiming that the cheese obtained had an excellent flavor (Almeida and Simões 2018). Starting from the second half of the 19th century, the evolution of rennet of animal origin led to a reduction in the use of vegetable coagulants; these, in fact, pass from a primary to a secondary role. Subsequently, interest in vegetable coagulants grew again, when there was a rapid increase in the consumption of cheese and a reduction in the availability of animal rennet (Shah et al. 2014). Proteases are enzymes present uniformly in the tissues of plants; they take on various functions from germination to senescence processes (Schaller 2004). Proteases are classified based on the amino acid residues involved in the catalytic site and are divided into cysteine, serine, aspartic, and metalloprotease (that possess a metal-ion cofactor in the catalytic site) (Mazorra-Manzano et al. 2018) Table 1 showcases the plant enzyme coagulants recently discovered with their strengths, weaknesses, and optimal pH/temperature parameters. The main proteases involved in dairy preparations belong to the first three groups and none from metalloprotease (Shah et al. 2014) and for some of them, the specific hydrolytic site is not yet available. Similar to chymosin, many vegetable proteases selectively hydrolyze the Phe₁₀₅-Met₁₀₆ κ -casein bond, while others hydrolyze different sites, such as protease extracted from *Solanum dubium* hydrolyses Ser₁₀₄-Phe₁₀₅ bond of bovine κ -casein while actinidin (protease from *Actinidia chinensis*), which probably hydrolyses the Arg₉₇-His₉₈ or Lys₁₁₁-Lys₁₁₂ bond (Ahmed et al. 2010; Lo Piero et al. 2011). Moreover, the extract from the ginger rhizome (*Zingiber officinale*) can hydrolyze κ -casein at two different sites: Ala₉₀-Glu₉₁ and His₁₀₂-Leu₁₀₃, instead of proteases from *Cynanchum otophyllum* Schneid hydrolyses specifically the Ser₁₃₂-Thr₁₃₃ bond (Jadhav et al. 2018; Luo et al. 2018). The hydrolysis of κ -casein, which is essential to initiate the coagulation process during

cheesemaking, directly affects the milk-clotting activity (MCA) of the enzyme, which is not influenced by the difference in the cleavage point (Drøhse and Foltmann 1989). On the other hand, the difference in hydrolysis specificity of the different proteases towards α - and β -casein contribute to the release of peptides that can influence the flavor and texture of the cheese (Zhang et al. 2019). For example, cardosin A (from *C. cardunculus*) acts on different bonds of α s1-casein: Phe₂₄-Phe₂₅, Arg₁₀₀-Leu₁₀₁, Phe₁₅₃-Tyr₁₅₄, Trp₁₆₄-Tyr₁₆₅, and Tyr₁₆₅-Tyr₁₆₆, also on β -casein: Leu₁₂₇-Thr₁₂₈, Leu₁₆₅-Ser₁₆₆, and Leu₁₉₂-Tyr₁₉₃ (Cavalli et al. 2013). The protease from cock's eggs (*Salpichroa organifolia*) hydrolyses the Phe₂₃-Phe₂₄ and Trp₁₆₄-Tyr₁₆₅ bonds in α -casein and the Leu₁₉₂-Tyr₁₉₃ bond in β -casein (Rocha et al. 2021). These different sites of action of the proteases lead to the formation of peptides that can have functional aspects, such as antihypertensive, immunomodulating, and antithrombotic activity (Almeida and Simões 2018). However, the general hydrolysis of caseins is related to the proteolytic activity (PA) of the enzyme. High PA may be associated with the development of bitter taste and cheese texture defects (Harboe et al. 2010). These properties and characteristics mainly depend on the activity of starter/adjunct culture, but also the type of enzyme used for cheesemaking influence them at the beginning of cheese ripening (Amira et al. 2017).

1.3.1 Aspartic Proteases

Aspartic proteases have two aspartic acid residues in the catalytic site, they involve a water molecule that acts as a nucleophile in the hydrolysis reaction (Mandujano-González et al. 2016). Most aspartic proteases consist of single-chain enzymes with a molecular weight of about 35 kDa and a length of about 330 amino acids (Szecsi 1992; Davies 1990). These types of proteases are most active at acid

pH (pH value 3–5) (Yegin et al. 2011) and consist of two lobes containing the aspartic acid residues mentioned above, which are essential for carrying out the catalytic function of the enzyme (Rawlings and Bateman 2009). Aspartic proteases have a structure that consists almost entirely of β -sheet and minimally of α -helix (Nugent et al. 1996). These proteases have been found in many vegetable tissues, such as: *Citrus aurantium* flowers (Mazorra-Manzano et al. 2013), *Withania coagulans* fruit (Salehi et al. 2017), and *Cirisium vulgare* flowers (Lufrano et al. 2012). The most widespread and used aspartic proteases are certainly the cardosins extracted from *C. cardunculus* (Verissimo et al. 1995). The most abundant are cardosin A and cardosin B; although, more recently, four additional cardosins have been isolated from flowers (cardosins E, F, G, and H) (Verissimo et al. 1995; Sarmiento et al. 2009). Cardosin A and cardosin B are heterodimeric glycoproteins composed of a heavy (31 and 34 kDa) and a light (15 and 14 kDa) chain. Cardosin A and B are often compared to chymosin and pepsin, respectively, as cardosin B is less selective than A in hydrolyzing caseins (Almeida and Simões 2018). However, a comparison between chymosin and cardosins shows that the latter have a greater PA, which leads to the onset of bitter flavors (Macedo et al. 1996; Sousa and Malcata 2002). In the Iberian Peninsula, the *C. cardunculus* extract containing these proteases is used to produce many cheeses that have a characteristic soft creamy texture and delicate flavor, sometimes slightly bitter but piquant when more mature (Roseiro et al. 2003). The industrial implementation of these proteases is inhibited by several factors such as the variability of these plant extracts in respect to enzymatic activity and the limited resources of flowers (Sousa and Malcata 2003). To overcome these limitations, the use of recombinant cardosins in *Escherichia coli* and in *Kluyveromyces lactis* has been studied (Almeida and Simões 2018). Tito et al. (2020) discovered two innovative aspartic proteases in

Solanum tuberosum capable of hydrolyzing milk caseins; specifically, the preferred substrate is represented by β -casein followed by α - and κ -casein. Both enzymes exhibited MCA in a dose-dependent manner with an optimum value determined at pH 5 and 30 °C. Furthermore, the different hydrolysis sites cleaved by *Solanum tuberosum* with respect to chymosin indicate the possible generation of peptides that could contribute to new tastes and aromas in cheese. In a similar way the aspartic protease contained in the fruits of *Salpichroa origanifolia*, (it has a maximum of MCA at 40 °C, pH 6.0) hydrolyzing mainly the α -casein and forming peptides that can have many potential biological activities (Rocha et al. 2021).

1.3.2 Cysteine Proteases

Cysteine-type proteases have Cys and His residues in their catalytic site and comprises of a total of 108 different families (Faheem et al. 2016). The active catalytic domain contains three catalytic residues (Cys-His-Asn) that have specific functions: the Cys residue acts as a nucleophile while the His residue acts as a general base for proton shuttling (Cstorer and Ménard 1994). These types of enzymes are produced as inactive precursors and have a peptide that acts as a signal for the secretion of the protein and an auto-inhibitory prodomain to prevent unwanted protein degradation (Coulombe et al. 1996). One of the most studied cysteine proteases is papain from *Carica papaya* (Konno et al. 2004), but other cysteine proteases have been extracted from ginger rhizomes (Hashim et al. 2011), from the root latex of *Jacaratia corumbensis* (Duarte et al. 2009), and from *Actinidia chinensis* (Puglisi et al. 2014). Albuquerque De Farias et al. (2020) found a cysteine protease present in the fruits of *Morinda citrifolia*. The enzyme extract of these fruits has an optimum temperature of 50 °C and a pH of 6.0. Cheeses produced with this

extract exhibited significantly higher fresh weight and yield than cheeses produced with commercial calf rennet with similar chewiness, but lower hardness, cohesiveness, elasticity, adhesiveness, and gumminess. A cysteine protease (MW 25.8 kDa) with MCA was isolated from *Dregea sinensis* stems with good activity and a wide pH range. However, it showed an optimum temperature of 80 °C, which is not used in dairy preparations (De Farias et al. 2020).

Finally, actinidin (EC. 3.4.22.14), is a cysteine protease abundant in kiwifruit, composed of 220 amino acid residues with a molecular mass of 23.5 kDa. This enzyme exhibited promising characteristics as a milk-clotting agent in cheese technology (Puglisi et al. 2014). There are many advantages derived from the use of actinidin as a coagulant enzyme in the cheesemaking: high MCA/PA ratio; the ability of specific hydrolyzing; and developing less off-flavor notes, attributed to bitter peptides (Serra et al. 2020). In addition, the preparation of an aqueous extract from kiwifruit is easier, faster, and cheaper than other plant coagulants (Lo Piero et al. 2011). Mazorra-Manzano et al. (2013) compared proteases extracted from ginger (*Zingiber officinale*) named zingibaine, melon (*Cucumis melo*) named cucumisin, and kiwifruit (*Actinidia deliciosa*) named actinidin, revealing that actinidin has the highest MCA/PA ratio compared to other vegetable coagulants. Moreover, Puglisi et al. (2014) showed that actinidin has an excellent MCA/PA ratio in production of mozzarella with kiwi juice as a coagulant without the onset of bitter flavors. The milk coagulation with actinidin created cheese with characteristics similar to that prepared with calf rennet. In fact, elasticity, cohesion, chewiness, and hardness of cheeses prepared using calf rennet and kiwi proteolytic enzyme extract were reported to be quite similar to each other. On the contrary, curds obtained using melon extracts had different textural properties as reflected by the low values of hardness, cohesiveness, chewiness, and springiness, possibly

due to the higher PA and the lower MCA/PA ratio (Mazorra-Manzano et al. 2013). Similar results were obtained from a recent study conducted by Fguiri et al. (2021), where the coagulating properties of kiwi extract were compared to those of ginger and pineapple extracts in camel (*Camelus dromedarius*) milk cheesemaking. The data reported that kiwi extract determined the highest yield (20.71%). Furthermore, the cheese made with the kiwi extract showed a better texture and had the highest scores in the test of sensory evaluation compared with other extracts.

1.3.3 Serine Proteases

Serine proteases are enzymes that involve a Ser residue in their catalytic site and are grouped into more than 20 families. Serine proteases form covalent enzyme/substrate complexes, they have strong nucleophilic amino acid residues in their catalytic site so perform a nucleophilic attack on the carbonyl group of the peptide bond of the substrate (Dunn 2001). They are present in many plant tissues, but more abundantly in fruits, and take part in many metabolic pathways of the plant (Rawlings and Barrett 1994). A serine protease capable of clotting milk was extracted from *Solanum dubium*, named dubiumin, which was reported to be very stable against a wide range of pH values (4.0–11.0) as well as a wide range of temperature (20–90 °C) (Ahmed et al. 2009). Other plant serine proteases capable of coagulating milk are cucumisin from *Cucumis melo* (Uchikoba and Kaneda 1996), religiosin from *Ficus religiosa* (Kumari et al. 2012) and streblin from *Streblus Asper* (Tripathi et al. 2011). An alternative to animal rennet can be represented by the serine protease extracted from fennel's (*Foeniculum vulgare*) tissues, this enzyme remains active and stable in the pH ranging from 6 to 7.5 and temperatures ranging from 40 to 60 °C (Bey et al. 2018). In many cases, raw extracts

from plants containing different types of proteases are used to coagulate the milk, this is the case of the extract of *Vallesia glabra*, where the different types of proteases influence the proteolytic profile of the extract in a very wide pH range (2.5–12.0) (González-Velázquez et al. 2021). The different tissues extract of crown flower (*Calotropis gigantea*) contains several proteases: results reported that latex exhibited high caseinolytic (86.45 U/mL) as well as MCA (450 U/mL) when compared to other parts (stem, flower, and leaf) (Anusha et al. 2014). *Bromelia pinguin* owns cysteine and serine proteases in the extract that are responsible for the major MCA in a broad temperature range with milk coagulation times comparable with commercial chymosin, but which also involve high PA; the MCA/PA ratio is 209 for chymosin and 1.29 for *B. pinguin*, respectively (Moreno-Hernández et al. 2017). Finally, *Balanites aegyptiaca* extract owes its proteolytic characteristics both to the class of aspartic proteases and to the serine class, which gives it two optimum pH values (pH 5.0 and pH 8.0, respectively) (Beka et al. 2014).

Table 1. Strengths, weaknesses and optimum pH/ temperature of plant proteases recently discovered. AP: Aspartic protease, CP: Cysteine protease, SP: Serine protease, NA: not available, NR: not revealed

Plant source	Tissues	Type	Strengths	Weaknesses	Temperature (°C)	pH	Reference
<i>Calotropis gigantea</i>	Latex, stem, flower and leaf	CP, SP	Latex has the highest MCA/PA ratio	high rate of proteolysis of crude enzyme	37	5.5	Anusha et al. (2014)
<i>Citrus aurantium</i>	Flower	AP	Raw extract of <i>Citrus aurantium</i> is capable of coagulating milk in similar times to those of animal rennet	NR	65-70	4.0	Mazorra-Manzano et al. (2013)
<i>Zingiber officinale</i>	Rizhomes	CP	The vegetable coagulant is easily extracted through few purification steps	Further studies are needed for industrial application	60	5.5	Hashim et al. (2011)
<i>Silybum marianum</i>	Flower	AP	Proteases extracted from <i>Silybum marianum</i> can clot both bovine, caprine and ovine milk proteins	NR	NA	NA	Cavalli et al. (2008)
<i>Balanites aegyptiaca</i>	Fruit	AP, SP	Milk-clotting activity was found from the extract from the fruit pulp	Further studies on the organoleptic acceptability of cheeses produced	50	5.0, 8.0	Beka et al. (2014)

<i>Cynara scolymus</i>	Flower	AP	Cheese yield is similar to that of animal rennet	are necessary 40 h of brine is necessary to avoid the development of bitter flavors in the cheese	40-60	4.0	Llorente et al. (2014)
<i>Foeniculum vulgare</i>	Stems	SP	The proteases are active at the temperature and pH parameters used for cheesemaking	The extraction process is complex	37	6.4	Bey et al. (2018)
<i>Dregea sinensis</i>	Stems	CP	Purified cysteine protease shows a wide range of activity (pH and temperature)	the optimum temperature is about 80°C which are not normally used in the cheese making process	80	6.0-9.0	Zhao et al. (2020)
<i>Bromelia pinguin</i>	Fruit	CP, SP	The enzyme extract is able to coagulate milk in a relatively short period of time	high caseinolytic activity after a long incubation period	45	2.5, 7.5	Moreno-Hernández et al. (2017)
<i>Morinda citrifolia</i>	Fruit	CP	With a milk clotting activity value of around 238.80 ± 5.29 U / mL, <i>Morinda</i>	slightly bitter taste but good acceptability of	50	6.0, 7.0	Albuquerque de Farias et al. (2020)

			citrifolia fruit extract proves to be a good candidate to replace calf rennet	cheeses				
<i>Vallesia glabra</i>	Leaf, fruits and seed	AP, CP, SP	The extract obtained from the leaves shows a great activity (0.20 MCU / mL) while in the fruits and seeds it was 0.12 and 0.11 MCU / mL, respectively	Further studies are needed to better characterize the wide variety of proteases present in the raw extract	65-70	4.0		González-Velázquez et al. (2020)
<i>Solanum tuberosum</i>	Tubers and leaves	AP	The two aspartic proteases are able to operate in optimal cheesemaking conditions (temperature 40–42 °C, pH values 6–6.2)	NR	30	5.0		Tito et al. (2020)
<i>Salpichroa origanifolia</i>	Fruit	AP	The activity of the enzyme allows to enrich the cheese with bioactive peptides deriving from the hydrolysis of α -, β -, and κ -casein which provide a health-promoting effect	NR	40	6.0		Rocha et al. (2021)
<i>Ficus johannis</i>	Latex	CP	low tendency to autolysis suggests probable use in industrial cheesemaking	Enzyme loses 20% activity at high salt concentrations	60	6.5		Afsharnejhad et al. (2018)

<i>Solanum elaeagnifolium</i>	Fruit	NA	This plant-derived protease is characterized by a good MCA/PA ratio	High concentrations of this coagulant can negatively affect the visco-elastic properties of the cheese	45	6.0	Gutiérrez-Méndez et al. (2019)
<i>Actinidia chinensis</i>	Fruit	CP	Kiwi extract exhibits high MCA/PA ratio compared to other plant coagulants	NR	40	5.5	Mazorra-Manzano et al. (2013)
<i>Cynara cardunculus</i>	Hairy root cultures	AP, SP, CP	<i>Cynara cardunculus</i> roots can produce proteases such as cardosin A with MCA, the phenotypic characteristic of high growth could lead to continuous supply from an application point of view	The low concentration of these proteases prevents industrial implementation	NA	NA	Folgado et al. (2021)

1.4 Proteolytic Activity and Bitter Taste by Plant Derived Coagulant in Cheese

The milk-clotting is essential to start the coagulation process; therefore, it is necessary to know how to evaluate the activity of each enzyme. To measure this parameter there are various methods such as the Berridge, the Soxhlet, and the International method, whose units of measurement are, respectively, Berridge units, Soxhlet units, and International milk-clotting unit (IMCU) (Harboe et al. 2010). As reported in Table 2, these methods differ from each other, and it is not easy to be able to compare the various units of measurement found in the literature (Harboe et al. 2010).

Table 2. Milk-clotting activity (MCA), proteolytic activity (PA), and MCA/PA ratio of plant extracts and other enzymes

Plant Extract	MCA	PA	MCA/PA	Reference
<i>Calotropis gigantea</i>	450 (U/mL)	86.45 (U/mL)	5.21	Anusha et al. (2014)
<i>Zingiber officinale</i>	314 (unit/mg)	0.19 (unit/mg)	1653.00	Hashim et al. (2011)
<i>Silybum marianum</i>	0.083 (RU/mL)	0.128 (EA/mL)	0.65	Cavalli et al. (2005)
<i>Balanites aegyptiaca</i>	2.43 (MCU/mL)	4.96 (MCU/mL)	0.49	Beka et al. (2014)
<i>Cynara scolymus</i>	147.65 (MCU/mg)	5.45 (Ucas/mg)	27.1	Llorente et al. (2004)
<i>Bromelia pinguin</i>	2.59 (U/mg)	2.0 (U/mg)	1.29	Moreno-Hernández et al. (2017)
<i>Morinda citrifolia</i>	238.8 (U/mL)	8.86 (U/mg)	27.00	Albuquerque de Farias et al. (2020)
<i>Vallesia glabra</i>	0.20 (U/mL)	19.04 (U/mL)	1.00	González-Velázquez et al. (2020)
<i>Ficus johannis</i>	21.88 (U/mL)	0.339 (IU/mL)	64.54	Afsharnezhad et al. (2018)
<i>Solanum</i>	4347.00 (U/mL)	1.3 (U-Gly/mg)	3343.00	Gutierrez-Mendez

<i>elaegnifolium</i>				et al. (2019)
<i>Actinidia chinensis</i>	2.7 (U/mg)	0.55 (U/mg)	5.00	Mazzorra-Manzano (2013)
Animal/microbial enzymes				
Calf chymosin	551.00 (SU/mg)	2.28 (U/mg)	243.20	Meng et al. (2021)
<i>R. miehei</i>	756.00 (SU/mg)	14.74 (U/mg)	51.31	Meng et al. (2021)

The high PA characterizes most of the vegetable proteases, which, especially during beginning of cheeses ripening, continue to hydrolyze the caseins causing the appearance of short peptides, which can have a bitter taste (Visser 1993). Many theories have tried to explain the origin of the bitter taste: i.e., the presence of hydrophobic amino acids inside the chain (Saha and Hayashi 2001); the presence in percentage of hydrophobic amino acids in the peptide side chains (Ney 1979). Specifically, the bitter taste is reduced when the hydrophobic amino acid is in a terminal position or it is completely free (Wieser and Belitz 1975). Ney gives a value to the average hydrophobicity of a peptide, called the Q value;

$$Q = \frac{\sum \Delta f}{n}$$

Δf represents the sum of the solubility data (i.e., the sum of the free energies of transfer of the amino acid side chains) divided by the number (n) of amino acid residues. When this value exceeds 1400, the peptide is to be considered bitter, while below 1300, the peptide is not bitter, but the bitterness of a peptide that has a Q value between 1300 and 1400 cannot be predicted (Ney 1979). Therefore, the presence of specific amino acids with high hydrophobicity values such as lysine, leucine, and proline in the side chains of the peptides leads to the onset of the bitter taste. In the caseins' micelles, there are large quantities of hydrophobic amino acids such as proline, valine, and leucine (Lee et al. 1996). As mentioned above, completely bond-free hydrophobic amino acids do not have a bitter taste, but the presence of proline-rich oligopeptides in the side chains confers bitter taste during cheese ripening (Tchorbanov et al. 2011). For this reason, the Q value turns out to be an important parameter for the identification of these peptides

that are generated by the extended proteolysis of clotting enzymes and other endogenous proteases even by starter proteases (Azarnia et al. 2003). Bitter peptides isolated from cheese are reported in **Table 3**. Hydrophobic amino acids are readily observed from both the C- and N-terminus of the peptide; all of them originated from α - or β -casein, as the result of secondary proteolysis (Lee et al. 1996).

Table 3. Bitter peptides from cheese

Origin	Peptide	Q-Value	Reference
α S1-CN (f11–14)	Leu-Pro-Gln-Glu	1367	Lee et al. (1996)
α S1-CN (f1–7)	Arg-Pro-Lys-His-Pro-Ile- Lys	1771	Lee et al. (1996)
α S1-CN (f191–197)	Lys-Pro-Trp-Ile-Gln-Pro- Lys	2010	Lee et al. (1996)
β -CN (f73–76)	Ile-Pro-Pro-Leu	2658	Toelstede et al. (2008)
β -CN (f60–68)	Tyr-Pro-Phe-Pro-Gly- Pro-Ile-His-Asn	1871	Toelstede et al. (2008)
β -CN (f8–16)	Val-Pro-Gly-Glu-Ile-Val- Glu-Ser-Leu	1390	Lee et al. (1996)
β -CN (f200–206)	Val-Arg-Gly-Pro-Phe-Pro	1718	Toelstede et al. (2008)
β -CN (f193–209)	Tyr-Gln-Glu-Pro-Val- Leu-Gly-Pro-Val-Arg- Gly-Pro-Phe-Pro-Ile-Ile-	1839	Singh et al. (2005)

Val

1.5 Effect of Plant Coagulants on Cheeses

Enzymatic coagulation of milk is a key step in the cheesemaking. The evaluation of enzyme activities of vegetable coagulants and their comparison with those of commercial rennet (chymosin) is an important first step in selecting a suitable rennet substitute. In this context, many efforts have been made to establish the influence of plant coagulants on rheological properties, sensory characteristics (texture, flavor, taste, and color) as well as yield of cheese. Most of the studies have been carried out on traditional cheeses type, mainly produced in the Mediterranean countries, in West Africa, and in Southern Europe, under traditional procedures in small dairies or farms (Roseiro et al. 2003). In Africa, there is an example of unripened cheese produced with the addition of a vegetable protease, the Warankashi, which is coagulated with the extract of Sodom apple leaf (*Calotropis procera*) (Hussein et al. 2016). Some Spanish, Portuguese, French, and Italian varieties of cheeses were produced with aqueous extracts of dried wild thistle flowers, of various species of the genus *Cynara* (Roseiro et al. 2003). This plant species grows spontaneously, especially in the south-west of the Mediterranean regions in arid and uncultivated soils. There are many Portuguese and Spanish cheeses that have the Protected Designation of Origin (PDO) and Protected Geographical Indication (PGI) certifications, available on the market that are produced using *Cynara cardunculus* dried flower extracts as a coagulant. These cheese types are: Serra and Serpa for Portugal; Los Pedroches, La Serena, Torta del Casar (from ewe's milk); and, also, Flor de Guía (from a blend of ewe's and goat's milk) from Spain (Roseiro et al. 2003). A typical example of cheese produced with *C. cardunculus* extract is Los Pedroches, which takes

its name from the area in the province of Cordoba (Spain). It is a hard, uncooked fatty cheese produced with raw Merino ewe's milk, coagulated with a vegetable coagulant, and ripened for about 2 months (Roseiro et al. 2003). During the cheese ripening, changes in the microbiological and physico-chemical parameters take place, giving a slightly spicy taste and a soft texture to the cheese (Fernández-Salguero and Sanjuán 1999). This is a clear example of how plant proteases hydrolytic activity over caseins have a significant effect on curd and cheese properties. However, long exposition to proteases can cause proteolytic degradation of the casein network (especially α - and β -casein), thus reducing approximately 0.3–0.7% of the curd yield (Jacob et al. 2011). Furthermore, the amount of protease used for cheesemaking is very important, because insufficient quantities lead to softer consistencies of the cheese while an excess of protease causes secondary proteolysis and, therefore, development of bitter flavors (Liu et al. 2021). This happens because in the secondary proteolysis, the nonspecific action of plant proteases against caseins produces peptides, which can be easily hydrolyzed into low molecular weight peptides (about 1400 Da) or into free amino acids by proteases of starter/adjunct cultures. In particular, low molecular weight peptides can have hydrophobic groups at C-terminal end, which give them a high Q value and therefore bitterness (Mazorra-Manzano et al. 2018; Lemieux and Simard 1991).

Indonesia produces Dangka, a cheese manufactured using papain (*Carica papaya*) as coagulant enzyme. The flavor of the cheeses prepared with different concentrations of papain was evaluated, and results clearly showed how the cheese obtained with a lower quantity of coagulant has a better flavor: each cheese was prepared using 2.5 L of milk, in which different amounts of papain were inoculated (0.06, 0.10, 0.14, and 0.16 g). The sample inoculated with 0.10 g of papain had the best results to the physical tests such as

flavor, color finish, body, and texture score, probably because lower quantities of coagulant are associated with a lower PA (Rana et al. 2017). Hence, the different hydrolysis rate of caseins by proteases can affect the cheese texture. According to the International Dairy Federation (IDF), cheeses have been classified based on their firmness into: extremely hard, hard, semi-hard, semi-soft, and soft cheese (Marino et al. 2019). **Table 4** shows the cheese texture in relation to the plant proteases used.

Table 4. Cheese types in relation to plant coagulant

Cheese Type	Name	Milk Type	Plant Coagulant Source	Reference
Soft	Torta del Casar	Ewe	<i>Cynara cardunculus</i>	Crespo et al. (2020)
	Dangke	Buffalo	<i>Carica papaya</i>	Hatta et al. (2013)
	Domiasi	Buffalo	<i>Heliantus hamnuus</i>	Darwish (2016)
	Warankashi	Cow and Soymilk	<i>Calotropis procera or Carica papaya</i>	Hussein et al. (2011)
	Castelo Branco	Ewe	<i>Cynara cardunculus</i>	Ferreira et al. (2009)
Semi-soft	Serra da Estrela	Ewe	<i>Cynara cardunculus</i>	Guiné et al. (2019)
	Serpa	Ewe	<i>Cynara cardunculus</i>	Araujo-Rodrigues (2020)
	Azeitão	Ewe	<i>Cynara cardunculus</i>	Cardinali (2021)
	La Serena	Ewe	<i>Cynara cardunculus</i>	Carbonell et al.

				(2002)
	Flor de Guía	Ewe and Goat	<i>Cynara cardunculus</i>	Iruzubieta et al. (2020)
	Mestiço de Tolosa	Ewe and Goat	<i>Cynara cardunculus</i>	Almeida et al. (2018)
	Caciofiore dei Sibillini	Ewe	<i>Cynara cardunculus</i>	Ricceri and Barbagallo (2016)
	Asadero	Cow	<i>Solanum elaeagnifolium</i>	Néstor et al. (2012)
Semi-hard	Los Pedroches	Ewe	<i>Cynara cardunculus</i>	Sanjuan et al. (2002)
	Évora	Ewe	<i>Cynara cardunculus</i>	Dias et al. (2021)
	Nisa	Ewe	<i>Cynara cardunculus</i>	Dias et al. (2021)

Parameters such as temperature range and enzyme concentration are fundamental in cheese manufacture (Beigomi et al. 2014). A percentage between 15% and 30% of the milk coagulant remains active in the curd and influences characteristics and flavors of cheese, even more than the indigenous bacterial microbiota. This is the case of *C. cardunculus* extract used for La Serena cheese giving soft consistency and a slightly bitter, sometimes spicy aftertaste (Mazorra-Manzano et al. 2018; Carbonell et al. 2002). For these reasons plant coagulants, such as those obtained from *C. cardunculus*, can suitably replace animal rennet in the production of soft cheeses such as Roquefort and Serra da Estrela (Roseiro et al. 2003). Therefore, although *Cynara cardunculus* proteases are suitable for all types of milk, cheeses prepared with cow's milk always develop more bitter flavors than those made with ewe's milk (Martínez-Ruiz et al. 2013). In fact, bovine and caprine caseins are more hydrolyzed by *C. cardunculus* extract than ovine caseins (Mazorra-Manzano et al. 2018). This is the main reason why, as shown in **Table 4**, many cheeses made with plant coagulant use ewe's milk as a substrate. However, the main issue remains in its high PA, which causes an intense bitter flavor, not always appreciated by consumers (Colombo et al. 2018). In a study of Martínez-Ruiz et al. (2013), the berries of *Solanum elaeagnifolium* (trompillo or silverleaf nightshade) in Chihuahua, northern Mexico, have been used in artisanal cheese named Asadero. Cheeses were obtained by a standardized process (32 °C milk temperature, 40 min renneting time), just changing the enzyme source. This type of cheese is softer than those made with chymosin due to their higher water content and proteolysis, and shows a shelf-life of 28 days at 4–6 °C. In addition, Domiati cheese in the work of Darwish (2016) was characterized by particular texture and flavor due to the action of vegetable proteases from *Abizia*

lebbeck and *Helianthus annuus* seeds. The cheeses made with these coagulants were prepared in milk heated to 50 °C and salted using sodium chloride to give a final concentration of 12%. The results showed that hardness, adhesiveness, gumminess, and chewiness of the cheeses produced with the plant coagulant were lower than those of control cheeses produced with chymosin. An important result obtained by Hashim et al. (2011) using ginger extract (*Zingiber officinale*) in the preparation of Peshawari cheese (semi-hard, fresh cheese made from whole or semi-skimmed buffalo or cow's milk). The cheeses obtained with the plant extract did not tend to be bitter and exhibited more appreciable sensory characteristics than those obtained with calf rennet, making ginger extract a promising substitute of animal coagulant. A similar result was obtained by Bruno et al. (2010) in the production of cheese with *Bromelia hieronymi* fruit extract that did not show bitter taste. Although the use of plant coagulants on an industrial scale is limited by some defects, a reorganization of the cheesemaking parameters could lead to the minimization of off-flavors to obtain a better quality of the final product (Rawlings and Bateman 2009).

1.6 Strategy to Improve the Use of Plant Derived Coagulant

One of the most important challenges of plant proteases is to overcome the problem of extract variability in composition, which involves a difficult standardization and limits the industrial implementation (Almeida and Simões 2018). The variability on composition derives from many factors such as the different location and composition of proteases within plant tissues (Barracosa et al. 2021).

For example, cardosin A accumulates more in the upper part of

the *C. cardunculus* flower, in the protein storage vacuoles of the stigmatic papillae, while cardosin B is distributed outside the cell wall and in the lower part of the pistil (Folgado and Abranches 2020). The concentration of cardosins increase with the senescence of the flower and the MCA of the extracts may depends on the plant ecotype used (Folgado and Abranches 2020; Correia et al. 2016). To reduce the variability on composition, the extraction/purification techniques such as “salting out” (i.e., the precipitation of proteins in solution by adding ammonium sulphate) coupled to chromatographic methods (for the subsequent purification of the desired enzyme) could be applied. This process allows to isolate the desired enzyme from the rest of the compounds present in the aqueous extract (sugars, vitamins, water, etc.) (Salehi et al. 2017; Duarte et al. 2009). Lyophilization is another approach to standardize plant aqueous extract. Recently a Spanish patent focused on the development of a powder extract of *C. cardunculus*, revealing that the extract becomes more hygienic, manageable, and stable during storage (Roseiro et al. 2003). Another solution is represented by the use of recombinant cardosin, being the best way to produce large quantities of these proteases at an industrial level, with best result for cardosin B (Almeida and Simões 2018). However, the characteristically low MCA/PA ratio of plant proteases produces undesirable effects in cheese, which can be managed in various ways. A possible strategy to improve the industrial use of vegetable coagulants could be the use of adjunct bacterial cultures with specific aminopeptidases, able to hydrolyze the bitter peptides formed during cheese ripening. Some lactic acid bacteria (LAB) such as strains belonging to *Lactococcus lactis* species, possess a complex proteolytic system capable of hydrolyzing the peptides, resulting in a bitter taste. From this point of view, the aminopeptidase N (PeP N) is one of the most important peptidases capable to hydrolyze peptides with hydrophobic N-terminal amino acid (Leu, Ala) (Christensen

1999). Other peptidases able to reduce bitter peptides formation responsible for bitter taste are: PepI, PepP, PepQ, PepR, and PepX. Each of these peptidases are able to hydrolyze the proline in a specific position within the peptide, reducing the bitter taste. These peptidases were already found in strains belonging to *Lactococcus lactis* and *Lactobacillus helveticus* (Navidghasemizad et al. 2013).

Another strategy to use plant coagulant with reduced bitter compounds formation is to ultrafiltrate cow's milk to keep a concentration in solids (fat, proteins, and minerals) similar to ewe's milk (Agboola 2002). In fact, the application of *Cynara cardunculus* proteases to ultrafiltered milk resulted in a semi-hard cheese with sensory properties similar to those of cheese made with animal rennet. Another method to reduce the defects caused by the excessive PA of the plant derived coagulating enzymes, could be represented by the use of both coagulating enzymes, with a high MCA/PA ratio, and techniques able to reduce the residual activity after milk-clotting. For example, one method is to apply high temperatures to the cheeses in order to inactivate the enzyme after milk-clotting. Unfortunately, this method is difficult to be applied because heat, besides denaturing the enzyme, halts the secondary proteolysis, and may cause a deterioration of cheese texture (Katsaros et al. 2018).

Furthermore, it is possible to apply HP (high pressure) to inactivate plant protease after milk coagulation. By applying a pressure of 600 Mpa combined with a moderate temperature of 40 °C for 35 min, cheeses without bitter flavors, unaltered color (Katsaros et al. 2018), and greater cohesiveness and hardness were obtained.

Finally, the modification of some cheesemaking and ripening parameters can allow avoiding the changes caused by plant coagulants. Lorente et al. (2014) showed how the parameters related to brining time can affect the flavor of the Gouda-type cheese using

the *Cynara scolymus* flower extract as milk coagulant. In detail, a curd salted in a brining NaCl solution of 22 °C for 40 h showed less bitter taste and organoleptic properties similar to cheeses manufactured with animal rennet. In fact, the high salt concentration allows the ionic strength to increase and, therefore, the hydrophobic interactions between the caseins to improve, thus inhibiting the hydrolysis of the hydrophobic regions of the caseins (such as C-terminal of β -casein) and releasing hydrophobic/bitter peptides (such as f193–209 from β -CN) (McSweeney 2007).

1.7 Conclusions and Future Perspective

The present review summarized the main plant coagulants used in cheese technology and their effects on various cheese types. Based on the literature, most plant-derived clotting enzymes showed high PA, resulting in bitter compounds in the final products. For this reason, plant coagulants still have limitations in cheese production. Raw milk and technological cheese parameters should be carefully chosen when plant coagulants are used. For instance, based on literature, ewe's milk is the most suitable for cheesemaking using plant derived proteases. Different sources of plant proteases (*Carica papaya*, *Ananas comosus*, *Ficus carica*, and *Cucumis melo*) with milk coagulating activity have been investigated. Unfortunately, these enzymes are characterized by high PA, and they can be used only for soft and creamy unripened cheese types. Among plant coagulants, actinidin is a promising rennet substitute for its specific hydrolytic site of action. Future researchers are required to better elucidate mechanisms involved in the casein hydrolysis and in peptide formation during the secondary proteolysis. The recent progress on recombinant form of some plant enzymes combined with more in-depth knowledge on the

biochemical mechanism could help to expand the portfolio of rennet substitute coagulants in the dairy industry, to satisfy the increasing market segment of Kosher and Halal consumers.

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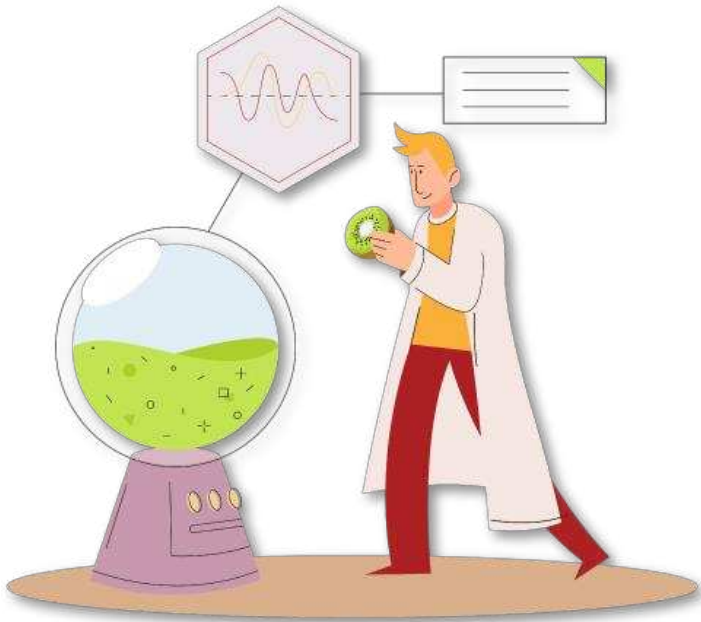
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Chapter 2



2 Chapter 2: An Easy and Cheap Kiwi-Based Preparation as Vegetable Milk Coagulant: Preliminary Study at the Laboratory Scale

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2.1 Abstract

In the present study, a kiwifruit aqueous extract was developed and used as a coagulant enzyme in cheesemaking. In detail, polyacrylamide gel electrophoresis (SDS-PAGE) was used to investigate the presence of actinidin, the kiwifruit enzyme involved in κ -casein hydrolysis, in different tissues (pulp, peel, and whole fruit) of ripe and unripe kiwifruits. Data revealed the presence of the enzyme both in the peel and in the pulp of the fruit. Although the aqueous extract obtained from the kiwifruit peel was able to hydrolyze semi-skimmed milk, it did not break down κ -casein. The aqueous extract obtained from the pulp showed a hydrolytic activity toward both κ -casein and semi-skimmed milk. The values for milk-clotting and proteolytic activity of the kiwifruit pulp extract were evaluated at different temperatures and pH parameters in order to obtain a high value of the MCA/PA ratio; we found that a temperature of 40 °C in combination with a pH value of 5.5 allowed us to obtain the best performance. In addition, the data revealed a higher hydrolytic activity of the enzymatic preparation from ripe kiwifruits than that from unripe ones, suggesting the use of the extract from pulp of ripe kiwifruits in the laboratory-scale cheesemaking. The data showed that 3% (v/v) of the ripe kiwifruit pulp extract determined a curd yield of 20.27%, comparable to chymosin yield. In conclusion, the extraction procedure for kiwifruit aqueous extract proposed in the present study was shown to be a fast, cheap, chemical-free, and ecofriendly technology as a plant coagulant for cheese manufacturing.

2.2 Introduction

Milk-clotting proteases are essential enzymes for cheesemaking, and among them, animal rennet, which is extracted from the abomasum of the newborn ruminants, is the most widely used. It contains a high amount of chymosin (EC 3.4.23.4), an aspartic protease that can hydrolyze a specific κ -casein bond (Phe₁₀₅-Met₁₀₆), thus causing the coagulation of milk during cheesemaking (Kumar et al. 2010). The worldwide increase in cheese production, combined with the reduction in supply and the increasing prices of calf rennet (García-Gómez et al. 2020), as well as religious components (in Islam and Judaism) and factors related to vegetarianism, have led to the search for alternative enzymes for coagulation of milk as appropriate substitutes for animal rennet (Colombo et al. 2021). Microbial coagulants are the most commonly substituted enzymes available; they are aspartic proteases produced by *Rhizomucor miehei* and *Rhizomucor pusillus* used to obtain various types of cheeses (Mamo and Assefa 2018). These enzymes have a low production cost, but also have some defects such as heat resistance, which involves a high proteolytic activity and therefore defects in the cheese (bitterness, low cheese yield) (Liu et al. 2021). The development of recombinant DNA technology allowed the creation of fermentation-produced chymosin (FPC) as an innovative substitute for animal rennet. This low-cost technology clones the bovine chymosin gene into a host microorganism that will produce it through fermentation (Dekker 2019), which obtains chymosin with the same characteristics of the animal one. By using EPS, it is possible to obtain cheese with excellent quality (Dekker 2019). However, technologies involving recombinant DNA are the subject of great debate for ethical reasons, and many countries, such as France, Germany and the

Netherlands, have restricted the use of FPC (Nicosia et al. 2022; Roseiro et al. 2003). Nowadays, a completely natural alternative is represented by plant-derived milk-clotting enzymes, which are commercialized and used as valid substitutes for animal rennet (Colombo et al. 2021). They have become of growing interest in the cheese industry due to their easy availability and simple purification processes. Among them, proteases are present in tissues of various plants, such as cardoon flower (Zikiou et al. 2020), *Cynara scolymus* artichoke (Bueno-Gavilá et al. 2020), ginger (*Zingiber officinale*) rhizomes (Hashim et al. 2011), *Dregea sinensis* stems (Zhao et al. 2021), and *Citrus aurantium* flowers (Mazorra-Manzano et al. 2013). Many of these enzymes have been widely used for the production of the Portuguese and Spanish soft cheese types. However, due the excessive proteolytic activity, plant-derived enzymes generate bitter flavors, limiting their industrial use. Among plant-derived enzymes, kiwifruit extract was revealed to have promising milk-clotting properties. Kiwifruit (*Actinidia deliciosa*) contains high amounts of actinidin (EC. 3.4.22.14), a cysteine protease, which showed a high potential for its use as a milk-clotting agent in cheesemaking (Puglisi et al. 2014). Actinidin is composed of 220 amino acid residues with a molecular mass of approximately 23.5 kDa (Carne and Moore 1978). The optimal parameters for actinidin activity, such as pH and temperature, are compatible with those used during cheesemaking. Actinidin and hydrolyze β -casein, followed by κ -casein at several points (Arg₉₇–His₉₈ or Lys₁₁₁–Lys₁₁₂ bonds), possess a higher specificity of hydrolysis against caseins than other proteases (such as papain) (Lo Piero et al. 2011). Mazorra-Manzano et al. (2013) demonstrated that cheese made with kiwifruit extract showed a higher dairy yield, chewiness, springiness, and gumminess than cheeses produced with melon (*Cucumis melo*) or ginger rhizomes (*Zingiber officinale*). In addition, kiwifruit extract revealed the highest

milk-clotting activity (MCA)/proteolytic activity (PA) ratio, thus representing the most effective alternative to calf rennet. However, the purification of kiwifruit extracts is a complex and time-consuming process, and often requires expensive equipment (Puglisi et al. 2014; Lo Piero et al. 2011; Mazorra-Manzano et al. 2013; Serra et al. 2020).

In the present study, a cheap, quick, and easy kiwifruit aqueous preparation was developed and used as a coagulant enzyme at the laboratory scale. The kiwifruit extract was obtained from both the pulp and peel of the fruit at different ripening times, and different extract concentrations of ripe fruit pulp were tested in a cheesemaking trial at the laboratory scale to establish the effect of the vegetable coagulant on the yield of the cheeses.

2.3 Materials and Methods

2.3.1 Raw Materials

The kiwifruits (*Actinidia deliciosa* cv. Hayward) used to obtain the aqueous extracts were purchased at a local market in Catania, Italy. The fruits were preliminarily evaluated for their ripeness degree using a digital refractometer (Atago, RX-5000, Tokyo, Japan): unripe (9.5° Brix) and ripe (14° Brix). Cow's milk was kindly provided by the La Cava dairy farm (Randazzo, Italy), and was used for cheesemaking at the laboratory scale.

2.4 Kiwifruit Aqueous Extracts Preparation

Unripe and ripe kiwifruits were washed, peeled with a knife, weighted, and pulped. The aqueous extract of the kiwifruit pulp was obtained by pressure using a manual stainless-steel press. The obtained juice was filtered twice through sterile gauze (50 grade, 20 × 20 cm) to separate all the seeds and the coarse content from the juice. The residual peel was blended (30 s) and then filtered through two

layers of sterile gauze to obtain the peel's aqueous extract.

Whole fruits were separately weighted, washed, blended (30 s), pressed (as above), and filtered to obtain whole-fruit aqueous extract following the same extraction procedure used for pulp. The kiwifruits were processed to obtain different aqueous extract preparations as follows: (i) ripe fruit pulp, (ii) ripe fruit peel, (iii) ripe whole fruit, (iv) unripe fruit pulp, (v) unripe fruit peel, and (vi) unripe whole fruit.

Aqueous extract yields of kiwifruits were expressed as mL of obtained juice per kg of whole kiwifruits processed. Finally, samples of 10 mL of each extract were placed in a convective oven at 105 ± 2 °C until reaching a constant weight (24 h) to determine the dry matter (DM) of the extracts.

2.4.1 Electrophoretic Analyses

An aliquot of all kiwifruit aqueous extract preparations was centrifuged for 10 min at 5000 rpm at 4 °C; then the supernatant was recovered and used for electrophoretic analyses. The protein content of extract samples was assessed using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Extract samples (2 µg in protein) were prepared for SDS-PAGE by adding an equal volume of loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue, and 5% 100 mM DTT).

The hydrolytic action of the aqueous extracts from the different tissues of ripe and unripe fruits toward κ -casein (Sigma, Darmstadt, Germany) and semi-skimmed cow milk was also evaluated. The hydrolysis was performed according to Puglisi et al. (2014). Partially skimmed milk and κ -casein (10 mg in protein) dissolved in 67 mM NaH_2PO_4 pH 7.2 buffer (final volume 300 µL) were used as substrates, and were incubated with each aqueous kiwifruit extracts (10 µg protein) for 20 min at 55 °C. Aliquots of 10 µL samples were loaded

into gel wells.

SDS-PAGE patterns (4–20% slab gels) were determined according to the method of Laemmli (1970). After the electrophoresis run was over (24 mA for 6 h), the gels were immersed for 8 h in a dye solution (50% methanol, 7.5% acetic acid, and 0.2% Coomassie Blue R-250). The excess dye was removed via several washings (for about 4 h) with a bleaching solution (15% methanol and 7.5% acetic acid).

2.4.2 *Milk-Clotting Activity Determination*

Based on the results obtained from the electrophoretic analysis, further tests were conducted only on the extract obtained from ripe kiwifruit pulp. The milk-clotting activity (MCA) was determined as described by Arima et al. (1970) with slight modifications. In detail, the aqueous extract obtained from pulp of ripe fruits was stabilized by adding an equal volume (v/v) of 20 mM sodium phosphate buffer (pH 7.0), then 1 mL of coagulant was added to 10 mL of low-fat (1%) pasteurized milk (containing 0.02% CaCl₂). The period elapsing between inoculation with the coagulant and the appearance of the first clot was calculated and expressed as the clotting time (seconds). The MCA was defined in terms of the Soxhlet unit (SU), representing the amount of protein in 1 mL of coagulant able to clot 1 mL of low-fat milk in 40 min (2400 s), and was expressed as: $MCA (SU) = 2400/t \times S/E$, where t = clotting time (sec), S = volume of milk (mL), and E = volume of extract (mL).

The MCA was tested at different temperatures (35 °C, 40 °C, 45 °C, 50 °C, and 55 °C) and pH values (5.2, 5.5, 6.0, 6.5, 7.0, and 7.5). The temperatures were settled using a thermostatic bath (WB-M50, Falc Instruments, Treviglio, BG, Italy), and the MCA was assayed at a constant pH of 7.0. The pH effect was monitored at the optimal temperature of 40 °C by adjusting milk samples at the

different pH values and monitoring with a digital pH-meter (MettlerDL25, Mettler-Toledo International Inc., Columbus, OH, USA). The assay was performed in triplicate.

2.4.3 Proteolytic Activity Determination

Proteolytic activity (PA) was determined using the method of Kunitz (1947) with low-fat milk powder as the substrate. Briefly, 50 μL of kiwifruit aqueous extract from the pulp of ripe fruits was added to 450 μL of 1% substrate solution (0.1 M phosphate buffer, pH 7.0) and incubated at 40 °C for 60 min. After incubation, the reaction was stopped by the addition of 500 μL of 5% (*w/v*) trichloroacetic acid (TCA); for the control sample, the TCA was added immediately before incubation, and then the sample was placed on ice. The mixture was vortexed (ZX3, Velp Scientifica, Usmate Velate, MB, Italy), left to stand on ice for 30 min, and then centrifuged at 13,000 rpm for 20 min. The optical density (OD) of the supernatant was then measured at 280 nm using a Perkin-Elmer Lambda 1 spectrophotometer. One unit of enzyme activity (U) was defined as the amount of protein that increased the absorbance by one unit at 280 nm under the conditions described above. PA was tested at different pH values (5.2, 5.5, 6.0, 6.5, 7.0, and 7.5) and at the optimum temperature of MCA (40 °C) using 0.1 M sodium phosphate buffer at the different pHs. The pH value was monitored using a digital pH meter (MettlerDL25, Mettler-Toledo International Inc., Columbus, OH, USA). The assay was performed in triplicate.

2.4.4 Cheesemaking at the Laboratory Scale

The cheesemaking was carried out at the laboratory scale following the method of *Cologna et al.* (2009) with slight

modifications, by placing Pyrex beakers in a thermostatic bath using 500 mL of cow milk at the optimal MCA temperature ($40^{\circ} \pm 1$). Once the temperature of 40°C was reached, lyophilized commercial starter cultures, provided by the La Cava farm, were added for milk acidification until reaching a pH of 5.5 ± 0.2 , the optimal pH for the highest MCA/PA ratio. Different amounts of aqueous extract, corresponding to 1.6, 2.0, 3.0, and 4.0% *v/v*, obtained from the pulp of ripe kiwifruits were added to the milk samples. When coagulation was completed, the curd was broken into very small irregular granules (4–6 mm) with a thorn, then pressed for 15 min in a cheese mold. The curd was turned and pressed for 30 min to facilitate the purging of the whey. Curd yields were calculated as curd weight/milk weight $\times 100$. Chymosin and microbial coagulant (supplied by Caglifacio Clerici, Como, Italy) were used as a control. The cheesemaking at the laboratory scale was conducted in triplicate.

2.4.5 *Statistical Analysis*

One-way ANOVA analysis with Tukey's post hoc test was applied in the MCA determination, PA determination, and cheesemaking test in three replicates using Statistica software (TIBCO Software, Palo Alto, CA, USA) to evaluate the statistical differences between the samples. Differences were statistically significant at $p < 0.05$.

2.5 *Results*

2.5.1 *Kiwifruit Aqueous Extracts Preparation*

The aqueous kiwifruit extract preparation was obtained by using the procedure described in **Section 2.3**, and showed extract yields of 364 mL/kg and 457 mL/kg from unripe and ripe kiwifruit

pulp, respectively. Similarly, yields of the aqueous extracts obtained from the whole ripe and unripe fruits were 469 mL/kg and 371 mL/kg, respectively, as shown in **Table 1**. Yields from the peels of both ripe and unripe fruits were low (15 mL/kg and 11 mL/kg, respectively).

Table 1. Aqueous extract yields from ripe and unripe kiwifruit tissues expressed as mL of obtained juice/kg of kiwifruit and as percentage of dry matter (DM) of the extract.

Tissue	Ripe		Unripe	
	Extract (mL/kg)	DM (%)	Extract (mL/kg)	DM (%)
Pulp	457 ± 2.5	17.5 ± 0.3	364 ± 3.1	14.5 ± 0.6
Peel	15 ± 2.1	12.4 ± 1.1	11 ± 1.8	10.8 ± 0.8
Whole fruits	469 ± 1.5	16.8 ± 0.4	371 ± 1.1	15.7 ± 0.9

2.5.2 SDS-PAGE Electrophoretic Profile

The presence of actinidin in the fruit tissues at different ripeness degrees was determined using electrophoresis; Figure 1 shows the pattern profiles of extracts from ripe fruit pulp (lane B), ripe fruit peel (lane C), ripe whole fruit (lane D), unripe fruit pulp (lane E), unripe peel (lane F), and unripe whole fruit (lane G). The results showed the presence of two main bands of approximately 20 kDa and 23 kDa in all tissue samples. The band of approximately 23 kDa corresponded to that of actinidin's molecular weight, as previously reported (Grozdanovic et al 2013). The hydrolysis electrophoretic patterns of κ -casein subjected to the enzymatic action of kiwifruit aqueous extracts are reported in Figure 2. The band with a molecular weight of 19 kDa, corresponding to κ -casein as reported by the producer (Sigma), was completely hydrolyzed by the aqueous extracts from the pulp of ripe fruits (Figure 2, lane D). Similarly, extracts from the pulp of unripe fruits (Figure 2, lane H) hydrolyzed κ -casein, but to a lesser extent than the extract from pulp of ripe ones. On the contrary, the aqueous extract from the peels of both ripe and unripe kiwifruits (Figure 2, lanes E and I) did not hydrolyze κ -casein, which remained intact after treatment, as indicated in Figure 2 (lanes E and I) by arrows. Finally, a partial hydrolysis of κ -casein due to the action of extracts from both ripe and unripe whole fruits was detected (Figure 2, lanes F and L). Figure 3 shows the patterns of semi-skimmed milk treated with the kiwifruit aqueous preparation. In all samples, hydrolysis of the milk proteins produced peptides showing an apparent molecular weight ranging from 8 to 9.6 kDa. These bands were less evident in milk treated with extracts from peel samples (Figure 3, lanes E and H), suggesting that a different degree of hydrolysis of the milk occurred. Furthermore, a band with a molecular weight of 16.9 kDa

was clearly detected in all samples.

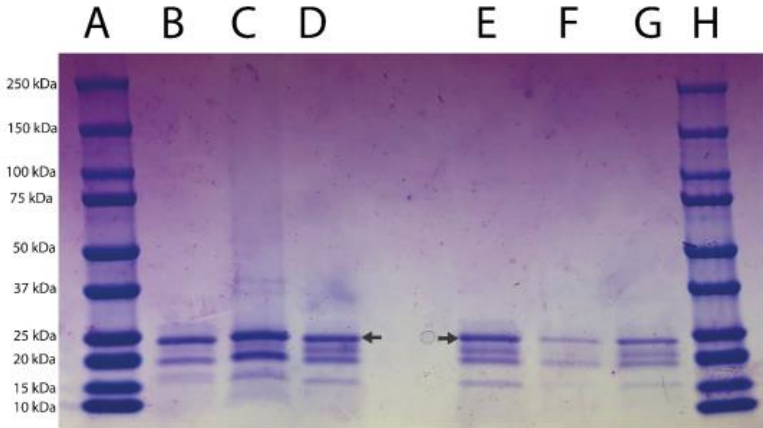


Figure 1. SDS-PAGE patterns of aqueous extract of ripe and unripe kiwifruits. Lanes A, H: mo-lecular markers; lane B: ripe fruit pulp; lane C: ripe fruit peel; lane D: ripe whole fruit; lane E: unripe fruit pulp; lane F: unripe fruit peel; lane G: whole unripe fruit. The arrows indicate actinidin bands corresponding to approximately 23 kDa.

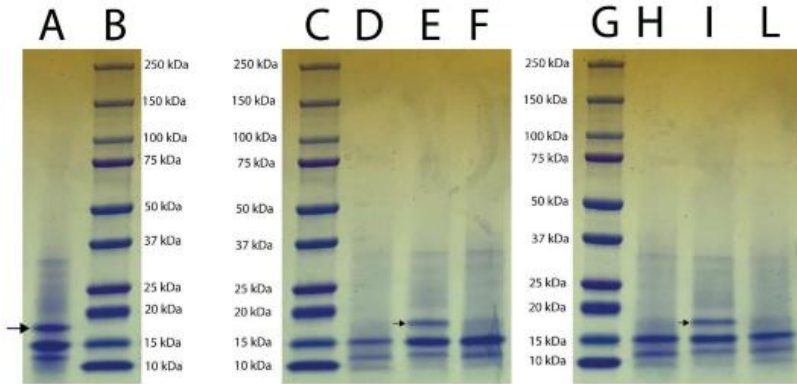


Figure 2. SDS-PAGE patterns of κ -casein subjected to treatment with aqueous

extracts of kiwifruit tissues. Lane A: control κ -casein; lanes B, C, G: molecular markers; lane D: ripe fruit pulp; lane E: ripe fruit peel; lane F: ripe whole fruit; lane H: unripe fruit pulp; Lane I: unripe fruit peel; lane L: whole unripe fruit. The arrows indicate the band corresponding to κ -casein (19 kDa, Sigma).

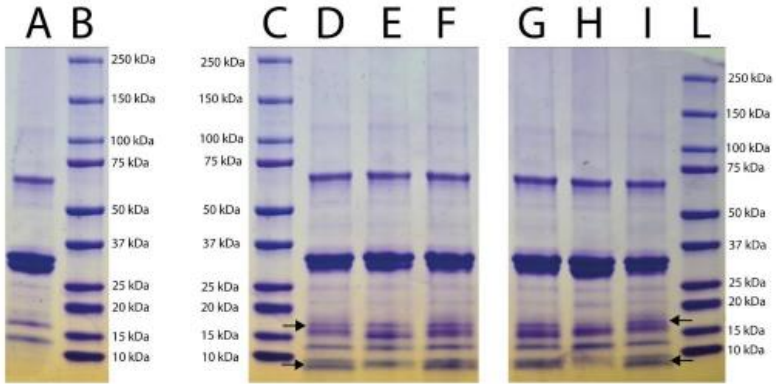


Figure 3. SDS-PAGE patterns of semi-skimmed milk treated with aqueous extract of kiwifruit tissues. Lane A: control semi-skimmed milk; lanes B, C, L: molecular marker; lane D: ripe fruit pulp; lane E: ripe fruit peel; lane F: ripe whole fruit; lane G: unripe fruit pulp; Lane H: unripe fruit peel; lane I: whole unripe fruit. Arrows indicate the peptides formed after milk hydrolysis.

2.5.3 *Temperature and pH Effects on Milk-Clotting Activity (MCA)*

Based on previous results, the aqueous extract from the pulp of ripe kiwifruits was used for further experiments. The effect of temperature on MCA is shown in **Figure 4**. The maximum (100%) MCA value (3.87 SU/mL) was reported at a temperature of 40 °C. The MCA slowly decreased when the temperature reached 45 °C (3.41 SU/mL), showing a drastic decrease at 55 °C (0.73 SU/mL). Furthermore, the effect of pH (ranging from 5.2 to 7.5) on MCA was evaluated, and the results are shown in **Figure 5**. The maximum (100%) value of MCA was detected at pH 5.5 (5.43 SU/mL), which

decreased at pH 6.0 and above.

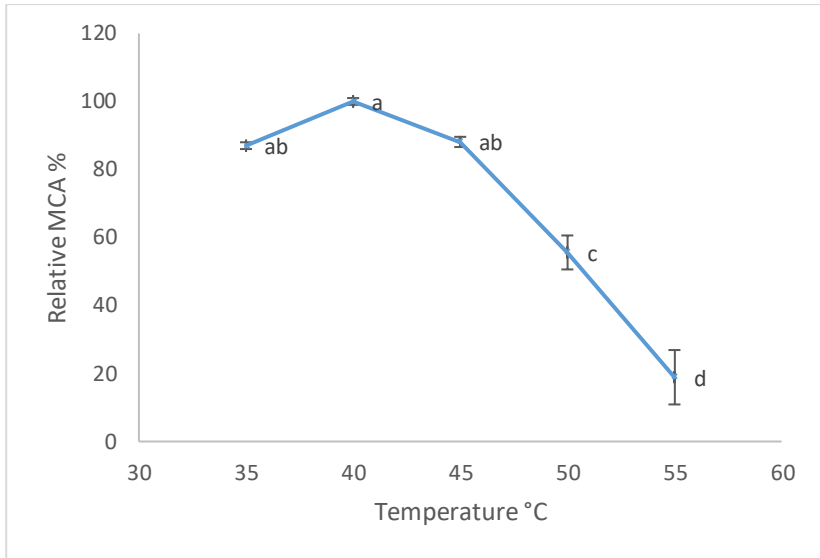


Figure 4. Temperature's effect on MCA of aqueous extract of pulp ripe kiwifruits. The percentage (%) of relative MCA represents the mean of three independent determinations performed in triplicate. The maximum value of MCA was 100%. Error bars represent standard deviations. Different lowercase letters (a, b, c and d) indicate a significant difference among samples at $p < 0.05$ (ANOVA with Tukey's post hoc test).

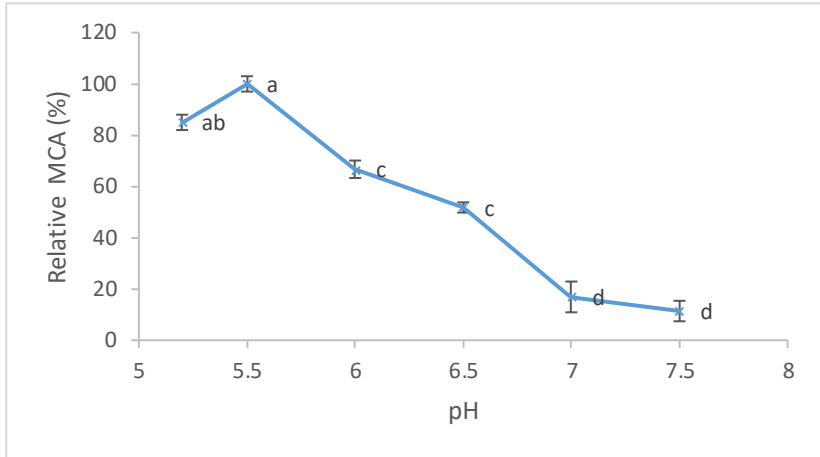


Figure 5. Effect of different pHs on milk-clotting activity of aqueous extract from ripe pulp ki-wifruits. The percentage (%) of relative MCA represents the mean of three independent deter-minations performed in triplicate. The maximum value of MCA was 100 %. Error bars represent standard deviations. Different lowercase letters (a, b, c and d) indicate a significant difference among samples at $p < 0.05$ (ANOVA with Tukey's post hoc test).

2.5.4 Effects of Different pHs on Proteolytic Activity (PA) and MCA/PA Ratio

The proteolytic activity was tested at different pH values (from 5.2 to 7.5) at a constant temperature of 40 °C (which was the best temperature previously determined for MCA), and results are shown in **Figure 6**. Proteolytic activity (PA) showed the maximum value (0.754 U/mL) at pH 6.5, then a slight decrease at a more acid or alkaline pH. At pH 5.5 (the best pH for MCA), a lower PA value was recorded (0.596 U/mL), suggesting that this pH may be a suitable value for an optimal condition for cheesemaking. Moreover, **Table 2** shows the MCA/PA ratio at different pH values.

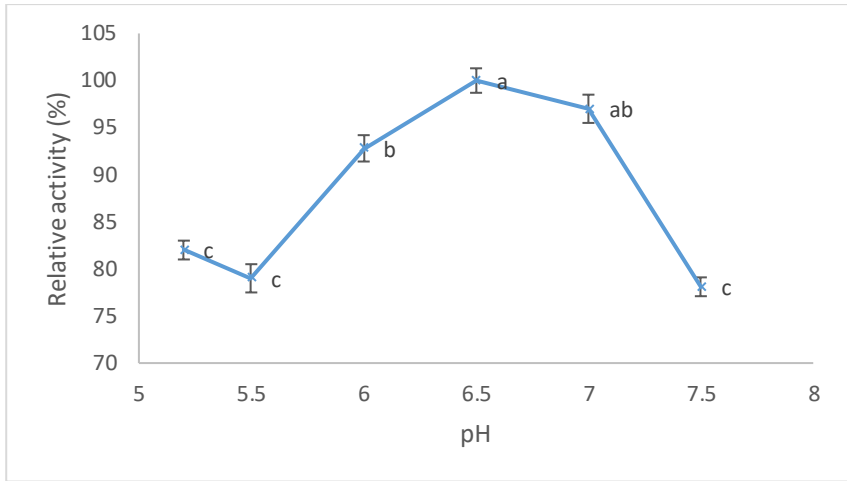


Figure 6. Effect of different pHs on proteolytic activity (PA) of aqueous extract from ripe pulp kiwifruit. The percentage (%) of relative PA represents the mean of three independent determinations performed in triplicate. The maximum value of PA was 100%. Error bars represent standard deviations. Different lowercase letters (a, b and c) indicate a significant difference among samples at $p < 0.05$ (ANOVA with Tukey's post hoc test).

2.5.5 Cheesemaking test

A cheesemaking trial at the laboratory scale was conducted using different amounts of the aqueous extract from the pulp of ripe kiwifruits. Table 3 reports the results for yields (%) of curds obtained from different percentages of the extract. In addition, the data were compared to those obtained from chymosin and microbial coagulant. The data revealed that a percentage (20%) of 3 (v/v) and 4 (v/v) of the kiwifruit extracts showed a comparable yield to those of chymosin and microbial coagulants.

Table 2. Effect of pH on MCA/PA ratio at 40 °C. MCA was expressed in SU/mL and PA was ex-pressed in U.

	pH					
	5.2	5.5	6.0	6.5	7.0	7.5
MCA/PA	7.43 ± 0.4 ^b	9.1 ± 0.5 ^a	5.1 ± 0.5 ^c	3.7 ± 0.9 ^c	1.3 ± 0.8 ^d	1.1 ± 0.5 ^d

Different lowercase letters (a, b, c and d) indicate a significant difference among samples at $p < 0.05$ (ANOVA with Tukey's post hoc test).

Table 3. Comparison between yield of curds obtained with chymosin, microbial coagulant, and different aliquots of aqueous extract from ripe kiwifruit.

Inoculum (v/v)	Ripe Aqueous Extract				Chymosin	Microbial
	4%	3%	2%	1.6%	0.04%	0.1%
Yield (%)	20.03 ± 1.84 ^a	20.27 ± 1.16 ^a	11.43 ± 1.96 ^b	10.90 ± 0.79 ^b	20.93 ± 0.90 ^a	20.06 ± 0.70 ^a

The values are the means of data from three replications. Data are reported as percentage value, and the standard deviation was calculated using three replications. Different lowercase letters (a and b) indicate a significant difference among samples at $p < 0.05$ (ANOVA with Tukey's post hoc test).

2.6 Discussion

Plant-derived enzymes have become of growing interest in dairy technology. Among them, actinidin, a cysteine protease from kiwifruit, is a promising substitute for chymosin due to its ability to form a good milk clot; moreover, the enzyme is fully compatible with technological parameters used during cheese manufacturing. Currently, several kiwifruit-extract preparation methods are already available in the literature (Puglisi et al. 2014; Lo Piero et al. 2011; Mazorra-Manzano et al. 2013; Serra et al. 2020; Raquib et al. 2022); however, they are time-consuming and high-cost procedures.

In the present study, a fast and cheap preparation of the aqueous extract of kiwifruit was developed and used for laboratory-scale cheesemaking. A higher yield in juice extract may be obtained by using ripe kiwifruits as a starting material, thus allowing the use of waste fruits with a high ripeness degree that are discarded by the food industry in the preparation of different kiwi-based foods and drinks. The data revealed that the kiwifruit aqueous extract from ripe fruit showed a higher clotting yield than that of unripe ones, suggesting a higher actinidin concentration in ripe fruit. It was noteworthy that the concentration of protein in kiwifruit is related to the growth stage, cultivar types, and treatment of the fruit during postharvest storage (Nishiyama and Oota 2002; Salzano et al. 2019). Our data from the SDS-PAGE profile suggested that the quantity of actinidin in the fruits' tissues was strongly dependent on the ripeness degree. These data were supported by other studies that demonstrated that plant coagulants, such as those prepared using Noni (*Morinda citrifolia* L.) fruits (De Farias et al. 2020) and berries of *S. elaeagnifolium* (Gutiérrez-Méndez et al. 2019), were obtained from ripe fruits. The hypothesis that the ripeness degree of the fruit positively influenced

the presence and the activity of the actinidin enzyme was also confirmed by Karki et al. (2018) (Karki and Ojha 2018) who found that the amount of actinidin was greater in ripe kiwifruits, which showed a higher level of activity (299 U/mg) against total casein with respect to unripe fruits.

In the present study, the presence of actinidin in the peel samples was also revealed, confirming the results of the study conducted by Nieuwenhuzen et al. (2007), in which the amount of actinidin was determined both in the pulp and in the peel of the fruits. However, although actinidin was detected in the peel samples, the hydrolysis electrophoretic pattern of the kiwifruit aqueous extracts toward κ -casein showed that extracts obtained from the peel of the fruits, both ripe and unripe, seemed unable to hydrolyze κ -casein (corresponding to the band at 19 kDa). The factors affecting hydrolysis activity toward κ -casein could be the presence of actinidin in the peel of the fruit, which was in a minimal quantity compared to the content present in the pulp, as reported by Lewis and Luh (1988); or an insufficient extraction of the target components from the peel during the procedure. Moreover, a lower hydrolysis pattern on κ -casein treated with the extract of whole kiwifruits was shown with respect to that obtained using pulp extract. This could be explained by the possible presence of substances in the peel of kiwifruits, which can determine a putative inhibition of κ -casein hydrolysis. The patterns of semi-skimmed milk treated with the aqueous extract of kiwifruits showed, in all the samples, bands of about 8–9.6 kDa, which were similar to those generated by the hydrolysis of semi-skimmed milk after the treatment with pure actinidin (Lo Piero et al. 2011). Moreover, according to Chalabi et al. (2014), bands corresponding to 16.9 kDa may be the result of hydrolysis produced by actinidin upon α -casein. These results were also comparable to those obtained by Puglisi et al. (2014), which showed a digestion product with a

molecular weight of 16.9 kDa generated by the hydrolytic action of an aqueous kiwifruit extract toward semi-skimmed milk. The aqueous extract from the pulp of ripe fruits showed an optimum temperature for MCA at 40 °C, in agreement with Mazorra-Manzano et al. (2013). This optimum temperature revealed that this preparation may be compatible with industrial cheesemaking. The MCA slowly decreased when the temperature reached 45 °C and 55 °C, probably due to the protease denaturation after the heat treatment. This result was comparable with that reported by Lo Piero et al. (2011), who demonstrated that when 55 °C was reached, the caseinolytic activity of actinidin was reduced by 30%. Finally, the maximum value of MCA detected at pH 5.5 was in agreement with results obtained by Grozdanovic et al. (2013) and Chalabi et al. (2014), who demonstrated that actinidin showed better performances at acid pHs ranging between 5.0 and 5.5.

The proteolytic activity (PA) of a clotting enzyme is an important parameter for cheese ripening; high values of proteolysis are often associated with the formation of off flavor and bitter taste due to the production of short peptides (Nicosia et al. 2022; Grozdanovic et al. 2013). The maximum value of proteolytic activity of the aqueous extract from the pulp of kiwifruits was reached at pH 6.5, and decreased at more acidic or alkaline pHs (**Figure 6**), in agreement with Lo Piero et al. (2011). Dehkordi et al. (2021) found that, although kiwifruit extract was a good substitute for animal rennet during cheesemaking, the use of this extract could lead to defects in the cheese (bitterness and soft texture) caused by its high proteolytic activity. Katsaros et al. (2009) inactivated the actinidin after coagulation of the cheese using high hydrostatic pressure (HHP) to reduce the defects caused by the high proteolytic activity of the kiwifruit extract. Another method that allows researchers to obtain a better cheese is to vary the parameters of pH and temperature in order

to manage the MCA/PA ratio, as high values of the MCA/PA ratio correspond to a coagulant capable of providing a cheese free of defects such as bitter flavors (Amira et al. 2017). In the present work, the highest calculated MCA/PA ratio was 9.1, and it was reached at 40 °C and a pH of 5.5. The aqueous extract at pH 6.0 reached an MCA/PA ratio of 5.2, which was similar to the ratio of 5.0 calculated by Mazorra-Manzano (2013).

The search for the suitable amount of coagulant is a critical point in cheesemaking, as an excess of proteases can influence secondary proteolysis (developing defects such as bitter flavors), while insufficient quantities lead to a loss in consistency of the cheese (Liu et al. 2021). Moreover, a long exposition to protease action can determine a proteolytic degradation of the casein network (especially α - and β -casein), thus reducing approximately 0.3–0.7% the curd yield (Jacob et al. 2011). In this work, curd made with an inoculum of 3% (*v/v*) of aqueous extract from pulp of ripe fruits provided a yield of 20.27%, and similar results were achieved when 4% of the coagulant was used (20.03%). These results suggested that an inoculum of about 3% (*v/v*) is enough to obtain a maximum curd yield that is comparable to the yields obtained using chymosin (20.93%) and microbial coagulant (20.06%). Lower yield values were found in similar studies. Mazorra-Manzano et al. (2013) used an extract of kiwifruit prepared from slices of peeled fruit stabilized by adding one equal part (*w/v*) of 20 mM sodium phosphate buffer (pH 7.2) and then homogenizing in a blender, obtaining a yield of 17.8% coagulated bovine low-fat milk. Ojha et al. (2021) reached a yield of 16.69% (goat milk) using a kiwifruit extract obtained after pressing the pulp and centrifuging it at 3000 rpm for 10 min as a coagulant. A statistical analysis showed that only the samples inoculated with 2% and 1.6% of the extract produced significantly different results, which meant that both amounts were not enough to reach an optimal yield.

The curd yield produced using 3% of the extract from the pulp of ripe kiwifruits was also comparable to those obtained from other plant extracts: the yields (bovine milk) obtained using latex from the plants of *Euphorbiaceae* family as a coagulant ranged from 20.73% (for *E. tirucalli*) to 21.30% (for *E. nerifolia*) (Mahajan and Chaudhari 2014). Furthermore, enzymes from sunflower used to coagulate cow milk showed a curd yield of 20.78% (Nasr et al. 2016). Finally, lower yields than that obtained using the preparation from pulp of ripe kiwifruits occurred when using several vegetable coagulants from berries of *Solanum elaeagnifolium* (17.77%) (Chávez-Garay et al. 2016), melon, and ginger (15.1% and 15.4%, respectively) (Mazorra-Manzano et al. 2013).

The high curd yield achieved in the present study may also have been related to the use of ripe kiwifruits and to the extraction method proposed, which was based on a fast and cheap procedure that was free of any added chemicals.

2.7 Conclusions

The extraction procedure for the kiwifruit aqueous extract proposed in the present study is a fast, cheap, -free, and ecofriendly technology to obtain an aqueous kiwifruit extract. The extract obtained from both the pulp and peel of the fruit at different ripening degrees exhibited different hydrolytic actions on κ -casein, suggesting that the actinidin concentration was influenced by the fruit ripening. Further studies on cheesemaking are ongoing to develop cheese types with desirable organoleptic and textual characteristics for industrial-scale cheese production.

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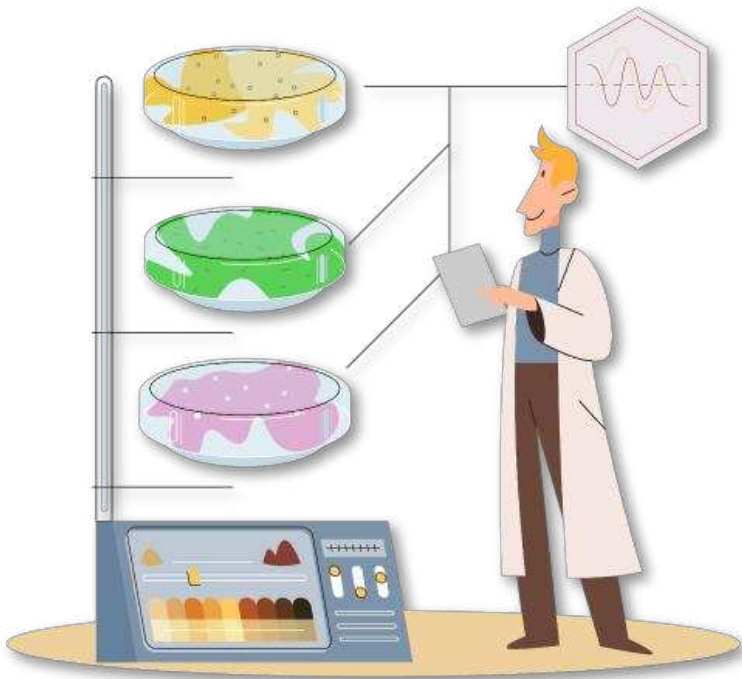
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Chapter 3



3 Chapter 3: Technological Characterization of Lactic Acid Bacteria Strains for Potential Use in Cheese Manufacture

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3.1 Abstract

A total of 26 lactic acid bacteria isolates from both Italian and Brazilian cheeses were tested for their use in cheesemaking. Isolates were screened for salt tolerance, exopolysaccharide and diacetyl production, lipolytic, acidifying, and proteolytic activities. In addition, the aminopeptidase (Pep N and Pep X) activities, were evaluated. Most of the strains demonstrated salt tolerance to 6% of NaCl, while only two *L. delbrueckii* (P14, P38), one *L. rhamnosus* (P50) and one *L. plantarum* (Q3C4) were able to grow in the presence of 10% (w/v) of NaCl. Except for 2 *L. plantarum* (Q1C6 and Q3C4), all strains showed low or medium acidifying activity and good proteolytic features. Furthermore, lipolytic activity was revealed in none of the strains, while the production of EPS and diacetyl was widespread and variable among the tested strains. Finally, regarding aminopeptidase activities, 1 *L. delbrueckii* (P10), 1 *L. rhamnosus* (P50), and 1 *L. lactis* (Q5C6) were considered as the better performing, showing high values of both Pep N and Pep X. Based on data presented here, the aforementioned strains could be suggested as promising adjunct cultures in cheesemaking.

3.2 Introduction

Lactic acid bacteria (LAB), normally present in milk, utensils, and surfaces of dairy farms, play an important role in cheesemaking, both in the early stages of milk fermentation and during cheese ripening (Walther et al. 2008; Carpino et al. 2017). In particular, the starter LAB (SLAB), rapidly fermenting the lactose, produces high concentrations of lactic acid whereas the non-starter LAB (NSLAB) is mainly involved during the ripening process in the definition of the sensory profile of the final product (Guarcello et al. 2016). The NSLAB carefully selected based on particular metabolic features and intentionally added as “adjunct cultures”, can improve the taste, flavor, and texture of cheese (El Soda et al. 2000). Three primary LAB metabolic pathways, including lactate and free fatty acid release metabolisms as well as proteolysis (with subsequent peptides and amino acid catabolism), are mainly involved in the definition of the sensory profile of the final product (McSweeney and Sousa 2000). In addition, by fermenting citrate, glucose, lactose, and other carbon sources, the produced diacetyl and acetoin confers desirable sensory features to ripened cheeses (McAuliffe et al. 2019; Pino et al. 2018). In particular, diacetyl plays a key role in the flavor development of Dutch-style cheeses, cottage cheese, quark, and many other fermented dairy products (Fox et al. 1995). Moreover, different studies demonstrated that some LAB provides an important contribution to the development of the texture of cheeses through, for example, the production of exopolysaccharides (EPS) (Kumari et al. 2020; Pino et al. 2019). As stabilizers, EPS can improve the firmness of the casein network by binding water and interacting with other milk components, such as whey proteins and casein micelles, along with promoting antimicrobial and antioxidant activities in dairy products (Korc and

Varga 2021). Furthermore, LAB possesses a complex enzymatic system that includes proteinases and peptidases. Peptidases are able to hydrolyze small peptides starting from long peptide chains, which in turn are freed from milk caseins by the action of coagulating enzymes and microbial proteinases themselves (Kunji et al. 1996). The accumulation of peptides with the presence of hydrophobic amino acids (lysine, leucine, and proline), in the peptide side chains, is generally associated with the development of a bitter taste in cheese (Lemieux and Simard 1991).

Some enzymes, produced by LAB, are able to selectively hydrolyze these peptides reducing the bitter taste. From this perspective, aminopeptidase N (Pep N; EC. 3.4.11.2) is one of the most significant peptidases able to hydrolyze peptides with hydrophobic N-terminal amino acids (e.g., Leu and Lys). Moreover, X-prolyl-dipeptidyl aminopeptidase (PepX; EC 3.4.14.11) is an additional peptidase with the ability to hydrolyze proline in specific peptide sites mitigating the bitter taste of the cheese (Nicosia et al. 2022). All these properties are important for the selection of an adjunct culture able to improve both the taste and texture of cheeses. All the technological properties mentioned above contribute to flavor development and quality of the cheese, which are strongly determined by the complex dynamics and interaction among cheese indigenous LAB (Randazzo et al. 2007). In particular, the indigenous LAB population arising from milk and dairy environment, usually consisting of streptococci, enterococci, and lactobacilli, is considered a source of enzymatic activity involved in flavor formation, especially in traditional cheeses (Randazzo et al. 2007).

The aim of this work was to characterize the technological features of LAB strains, isolated from different traditional cheeses, in order to set up a starter or an adjunct culture for cheesemaking. In particular, indigenous strains were isolated from Pecorino Siciliano

and from Ragusano cheeses which are traditional Italian hard cheeses manufactured from raw ewe's and cow's milk, respectively, without any starter cultures, using ancient manufacturing processes and ripened at least for 4–6 months or longer (Carpino et al. 2017; Randazzo et al. 2008). Brazilian strains were isolated from Marajó cheese, a traditional Brazilian cheese produced in the Marajó island, made from raw buffalo milk, in accordance with the region's cultural traditions by spontaneous coagulation, and characterized by a complex flavor mainly generated by autochthonous LAB. This cheese has been produced for more than a century and is the socioeconomic foundation of tiny farmers on the island (Kamimura et al. 2019). All strains were tested for the ability to exert lipolytic and proteolytic activities, produce exopolysaccharides and diacetyl, and tolerate different NaCl concentrations. In addition, the presence of specific aminopeptidases (Pep N and Pep X), able to hydrolyze bitter peptides, was in-depth evaluated.

3.3 Materials and Methods

3.3.1 Bacterial Strains and Culture Conditions

A total of 26 LAB strains were screened in the present work in order to be selected as starter or adjunct culture. In detail: 11 belonging to the collection of InovaLeite (Laboratory of Milk and Dairy Products, Universidade Federal de Viçosa, Brazil) were isolated from Marajó and Pará cheeses using M17 agar medium (Difco laboratories, Ditroit, MI, USA) and MRS agar medium (Difco laboratories, Ditroit, MI, USA), and 15, belonging to the collection of the Laboratory of Microbiology (Department of Agricultural, Food, and Environment, Di3A, University of Catania, Italy), were isolated from Pecorino and Ragusano cheeses using Rogosa agar medium (Oxoid Ltd., Basingstoke, UK) and MRS agar medium (Oxoid Ltd., Basingstoke,

UK). The strains were previously identified at species level through molecular methods as follows: 4 *Lactiplantibacillus plantarum* (Q1C6, Q3C4, Q5C9, and Q6C4), 6 *Pediococcus acidilactici* (Q1C8, Q3C1, Q3C3, Q6C1, Q6C5, and Q22C2), 1 *Lactococcus lactis* subsp. *lactis* (Q5C6), 14 *Lactobacillus delbrueckii* (P7, P9, P10, P11, P12, P13, P14, P15, P33, P36, P37, P38, P39, and P40) and 1 *Lacticaseibacillus rhamnosus* (P50). The strains were stored in de Man Rogosa and Sharpe (MRS) broth (Oxoid Ltd., Basingstoke, UK) with 20% (v/v) glycerol and kept at -20 °C till further use.

3.3.2 Bacterial Growth and Cell Suspension Standardization

LAB isolates were cultured in MRS broth (Oxoid Ltd., Basingstoke, England) and incubated at 30 °C for 24 h. After growth, cells were centrifuged at 12,000 rpm for 2 min at 4 °C and the obtained pellet was suspended in NaCl (0.9% w/v) to a turbidity equivalent to the 1 McFarland (about 3×10^8 CFU/mL). The standardized cell suspension was used to test proteolytic, lipolytic, and acidifying activities, diacetyl and exopolysaccharides production as well as salt tolerance.

3.3.3 Proteolytic and Lipolytic Activities

The extracellular proteolytic activity was evaluated following the protocol described by Randazzo and co-workers (2021) and Franciosi and co-workers (2009). In detail, 2 µL of cells, standardized as previously described, were spotted on the surface of petri dishes containing Plate Count Agar (PCA, HiMedia, Mumbai, MH, India) supplemented with skim milk (10% w/v, Oxoid Ltd., Basingstoke, UK). After incubation at 30 °C for four days, plates were checked for

the presence of a clear zone surrounding the colonies.

The lipolytic activity was tested by spotting 2 μL of standardized cells (about 3×10^8 CFU/mL) on the lipolytic medium composed of peptone, 2.5 g/L; casein peptone, 2.5 g/L; yeast extract, 3 g/L; Agar, 12 g/L; and 1% of tributyrin. After incubation at 30 °C for 72 h, the presence of a halo around the bacterial spot revealed lipolytic activity (Hantsis-Zacharov and Halpern 2007).

Each test was performed in triplicate.

3.3.4 *Diacetyl and Exopolysaccharide Production*

In order to measure the diacetyl production, 0.1 mL aliquots of standardized cell suspension were added to 10 mL of sterile reconstituted skim milk (10% w/v, Oxoid Ltd., Basingstoke, UK), and the mixture was incubated at 30 °C for 24 h. Then, an aliquot of 1 mL was transferred to a sterile tube and supplemented with 0.5 mL of α -naphthol (1% w/v) and KOH (16% w/v), then the mixture was incubated at 30 °C for 10 min. Diacetyl production was indicated by the formation of a pink ring and classified as weak (+), medium (++), or strong (+++) according to the color intensity.

The feature relative to the synthesis of exopolysaccharide (EPS) was assessed using the methodology described by Dal Bello et al. (2012). In detail, 0.1 mL aliquot of the standardized cell suspension was inoculated into 10 mL of sterile reconstituted skim milk (10% v/v, Oxoid Ltd., Basingstoke, UK) and the mixture was incubated at 30 °C for 48 h. The presence of stringiness was used to evaluate the EPS production.

Each test was performed in triplicate.

3.3.5 *Salt Tolerance*

The ability of the tested isolates to grow at different salt concentrations was evaluated as described by Ferrari et al. (2016). MRS broth medium (Oxoid, Milan, Italy) containing bromocresol purple (0.04% w/v) and different NaCl concentrations (2, 6, 10% w/v) was transferred into sterile tube and inoculated with 1% of standardized cell suspension. After incubation at 37 °C for one week, the change of color from purple to yellow was considered as positive growth.

The test was performed in triplicate.

3.3.6 Acidifying Activity

Acidifying activity was determined by inoculating the standardized cell suspension (2% v/v) into reconstituted skim milk (10% w/v, Oxoid Ltd., Basingstoke, UK). The pH changes (Δ pH) were determined after 6 h and 8 h of incubation at 37 °C using a pH meter (MettlerDL25, Mettler-Toledo International Inc., Columbus, OH, USA).

The test was performed in triplicate.

3.3.7 Aminopeptidase Activity

3.3.7.1 Cell Free Extract Preparation

LAB strains were grown at 37 °C in MRS broth medium (Oxoid, Milan, Italy) until reaching the late exponential phase. After centrifugation (10.000 g, 10 min, 4 °C), cells were washed twice with sodium phosphate buffer (0.05 M, pH 7.0), then standardized to 10⁹ CFU/mL in the same buffer (0.05 M, pH 7.0). To obtain the cell free extract (CFE), cell lysis was carried out through the bead-beating

method, in the Precellys apparatus (Bertin technologies, Düsseldorf, Germany), using zirconium beads with a diameter of 0.1 mm. The treatment at 6000 rpm for 40 s was repeated twice. Samples were placed on ice (for 4 min) between each cycle. At the end of the treatment, the samples were placed on ice for 10 min. CFE was obtained after removing zirconium beads, cell debris, and unbroken cells by centrifugation (10.000 g, 10 min, 4 °C). The protein concentration was determined by using the Pierce™ BCA Protein Assay Kit (Thermo Fisher, Waltham, MA, USA).

3.3.7.2 Aminopeptidase N Activity

Aminopeptidase activity of the CFE was determined as described by Requena et al. (1993). Then, 100 µL of CFE were added to 80 µL of phosphate buffer (50 mM, pH 7.0) and 20 µL of a reaction buffer containing the substrate Lys-p-nitroanilide dihydrobromide (20 mM in methanol) (Sigma-Aldrich, St. Louis, MO, USA). After incubation at 37 °C for 30 min (or until the mixture reaches a strong yellow color) the reaction was stopped by adding 500 µL of glacial acetic acid (10% v/v, Panreac, Barcelona, Spain). Optical density (OD) at 410 nm was measured using iMark™ Microplate Absorbance Reader (Biorad, Milan, Italy). The test was performed in triplicate, using the “white test” (20 µL of the reaction buffer containing the substrate Lys-p-nitroanilide dihydrobromide with 180 µL of phosphate buffer) as blank. The aminopeptidase N (Pep N) activity was expressed as U/mg of protein. One U was defined as the amount of enzyme required to release 1 µmol of p-NA per minute under the assay conditions.

3.3.7.3 Aminopeptidase X Activity

For the evaluation of aminopeptidase X (Pep X), 50 μL of CFE were added to 600 μL of phosphate buffer (50 mM, pH 7.2) and 50 μL of a reaction buffer containing the substrate H-Ala-Pro-p-nitroanilide HCl (20 mM in methanol) (ChemCruz Biochemicals, Santa Cruz, CA, USA). After incubation at 37 $^{\circ}\text{C}$ for 30 min (or until the mixture reaches a strong yellow color), the reaction was stopped by adding 500 μL of glacial acetic acid (10% v/v, Panreac, Barcelona, Spain). Optical density (OD) at 410 nm was measured using iMark™ Microplate Absorbance Reader (Biorad, Milan, Italy). The test was performed in triplicate, using the “white test” (50 μL of a reaction buffer containing the substrate H-Ala-Pro-p-nitroanilide HCl with 150 μL of phosphate buffer) as blank. Specific activity was expressed as U/mg of protein. One U was defined as the amount of enzyme required to release 1 μmol of p-NA per minute under the assay conditions.

3.3.8 Statistical Analysis

Pep N and Pep X data were subjected to One-way ANOVA analysis with Tukey’s post hoc test using the Statistica software (TIBCO Software, Palo Alto, CA, USA). Differences were considered statistically significant at $p < 0.05$. In order to correlate acidifying and aminopeptidase activities, data were subjected to principal component analysis (PCA) using the XLSTAT (2023.1.1.1397) software.

3.4 Results and Discussion

3.4.1 Proteolytic and Lipolytic Activities, Diacetyl and Exopolysaccharide Production

Results of proteolytic and lipolytic activities as well as diacetyl and exopolysaccharide production are shown in **Table 1**. Overall, all the tested strains, with the exception of two *L. plantarum* strains

(Q1C6 and Q3C4), showed proteolytic activity. It is well known that LAB possesses an efficient proteolytic system, with complex combinations of proteinases and peptidases, which allow them to obtain organic nitrogen from complex proteins like casein (Liu et al. 2010). Proteases hydrolyze caseins forming peptides which, crossing the cell membrane through specific transport proteins, are further degraded into amino acids by intracellular peptidases (Kieliszek et al. 2021). The amino acids, through specific catabolic pathways, are transformed into volatile and non-volatile compounds, which play a key role in the definition of the sensory properties of cheese (Lawlor et al. 2002).

Concerning lipolytic activity, as reported in [Table 1](#), none of the strains exhibited this feature. Identical results were reported by Meng et al. (2018) and Monfredini et al. (2012). In fact, none of the tested LAB strains showed lipolytic activity through tributyrin agar, while Silva et al. (2020) found lipolytic activity only in 5 strains of LAB out of 37 tested. The inability of LAB strains to break down milk fat during ripening makes them suitable as adjunct cultures. In fact, it is well known that NSLAB should not possess lipolytic activity since this is associated with the development of rancid flavor (Tsigkrimani et al. 2022; Carafa et al. 2015; Herrero et al. 1996). Differently, one important feature of adjunct cultures is related to diacetyl production. Out of the 26 strains, 20 showed diacetyl production (**Table 1**); the strains isolated from Pecorino cheese were classified as medium diacetyl producers whereas LAB strains from Ragusano, Marajó, and Pará cheeses showed variable ability to produce diacetyl (**Table 1**). In detail, 4 *L. delbrueckii* (P11, P12, P13, and P39) and one *P. acidilactici* (Q3C1) strains displayed strong production, 4 *L. delbrueckii* (P7, P9, P10, and P36) 2 *L. plantarum* (Q5C9 and Q6C4), and one *L. lactis* (Q5C6) had medium production, 3 *P. acidilactici* (Q1C8, Q6C5 and Q22C2), 2 *L. plantarum* (Q1C6 and

Q3C4), one *L. delbrueckii* (P38), and one *L. rhamnosus* (P50) strains showed weak production, while 4 *L. delbrueckii* (P14, P15, P33, and P40) and 2 *P. acidilactici* (Q3C3 and Q6C1) strains had no production. This wide variability in diacetyl production was previously observed in *L. rhamnosus* strains isolated from semihard goat cheese (Meng et al. 2018), and in *L. rhamnosus* and *L. delbrueckii* subsp. *bulgaricus* strains isolated from hard raw cow's milk cheese (Monfredini et al. 2012). This evidence is in accordance with previously reported data, suggesting that diacetyl production is a strain-dependent feature (Franciosi et al. 2009; Câmara et al. 2019). The production of this compound represents a very important variable in the choice of a strain for the formulation of an adjunct culture; in fact, the diacetyl, produced by LAB using citrate, glucose, lactose, and other carbon sources as substrates, confers a buttery taste to dairy products (Peralta et al. 2017; Rincon-Delgado et al. 2012). Furthermore, diacetyl was shown to have inhibitory activity against foodborne pathogens, especially when combined with bacteriocin such as nisin (Thierry et al. 2015).

Concerning EPS production, three *L. delbrueckii* strains (P10, P14, and P38) isolated from Pecorino and Ragusano cheeses showed the ability to produce these compounds (**Table 1**). The results are in agreement with those previously reported by Christianah et al. (2008) and Khubaib et al. (2018), showing that strains belonging to *L. delbrueckii* and *L. delbrueckii* subsp. *bulgaricus* were able to produce EPS and among them, *L. delbrueckii* subsp. *bulgaricus* is a well-known EPS producer (Zhu et al. 2019). In detail, *L. delbrueckii* subsp. *bulgaricus* exhibit many intraspecific biosynthetic pathways producing different EPS structures, consisting of units of repeated monomers such as glucose, galactose, rhamnose, and sometimes fructose (Shene and Bravo 2007). *L. delbrueckii* subsp. *bulgaricus* EPS producers have been used for

many years in classical yogurt, drinking yogurt, fresh cheeses, cultured cream, or milk-based desserts, thanks to their ability to increase viscosity, prevent syneresis and improve sensory and nutritional characteristics of dairy products (Bancalari et al 2022; Xu et al. 2019). In the work of Ahmed et al. (2005), the employment of an EPS-producing culture, during the manufacture of the Karish cheese, strongly influenced the textural properties of the final product. The resulting cheese exhibited lower hardness, consistency, chewiness, and adhesiveness compared to the cheese obtained using a strain unable to produce EPS. Furthermore, the panelists described the cheese made with the EPS-producing strain as smooth, creamy, moist, and soft, while the one with the EPS-nonproducing strain was described as dry and granular. In addition, several functional features were recently attributed to EPS produced by LAB, such as antitumorigenic, antimicrobial, and antioxidant activities. In particular, EPS are able to counteract reactive oxygen species (ROS) hindering the development of many disorders including lung injury, atherosclerosis, inflammation, aging, and cancer (Saleem et al. 2021).

Table 1. Identification and technological properties of strains isolated from Pecorino cheese, Ragusano cheese, Marajó cheese and Pará cheese.

Isolate	Isolation Source	Species attribution	Proteolysis*	Lipolysis*	Diacetyl**	EPS*	Accession n. ^a
P7, P9	Pecorino Cheese	<i>L. delbrueckii</i>	+	-	++	-	MT459343.1
P10	Pecorino Cheese	<i>L. delbrueckii</i>	+	-	++	+	OQ180823
P11, P12, P13, P39	Ragusano Cheese	<i>L. delbrueckii</i>	+	-	+++	-	OQ180823
P14	Ragusano Cheese	<i>L. delbrueckii</i>	+	-	-	+	OQ180823
P15, P33, P40	Ragusano Cheese	<i>L. delbrueckii</i>	+	-	-	-	MT459339.1
P37	Ragusano Cheese	<i>L. delbrueckii</i>	+	-	+	-	MT464341.1
P36	Ragusano Cheese	<i>L. delbrueckii</i>	+	-	++	-	KE145374.1
P38	Ragusano Cheese	<i>L. delbrueckii</i>	+	-	+	+	KE145374.1
P50	Ragusano Cheese	<i>L. rhamnosus</i>	+	-	+	-	OQ180824
Q5C9, Q6C4	Marajó Cheese	<i>L. plantarum</i>	+	-	++	-	AP018405.1

Q1C6, Q3C4	Marajó Cheese	<i>L. plantarum</i>	-	-	+	-	AP018405.1
Q1C8, Q6C5, Q22C2	Marajó Cheese	<i>P. acidilactici</i>	+	-	+	-	CP015206.1
Q3C1	Marajó Cheese	<i>P. acidilactici</i>	+	-	+++	-	CP015206.1
Q3C3, Q6C1	Marajó Cheese	<i>P. acidilactici</i>	+	-	-	-	CP015206.1
Q5C6	Pará Cheese	<i>L. lactis</i>	+	-	++	-	CP033606.1

*(+), positive; (-), negative.

**(+++), strong production; (++) medium production; (+), weak production; (-), no production.

^aAccession number of sequence of closest relative found with Blast search.

3.4.2 Salt Tolerance and Acidifying Activity

As reported in **Table 2**, all tested strains were able to grow in presence of 2% (w/v) of NaCl. Moreover, 13 strains (7 *L. delbruekii*, 2 *L. plantarum*, 3 *P. acidilactici* and one *L. lactis*) demonstrated salt tolerance at the concentration of 6% (w/v), and a total of 4 strains (2 *L. delbruekii* (P14 and P38), 1 *L. rhamnosus* (P50) and 1 *L. plantarum* (Q3C4) grew in presence of 10% (w/v) of NaCl. The results agree with the data reported by Meng et al. (2018), which demonstrated the ability of *L. rhamnosus* strains isolated from semihard goat cheese to grow in presence of high NaCl concentration (10% w/v). In addition, *L. rhamnosus* isolates from traditional Provola dei Nebrodi cheese showed high tolerance to the presence of NaCl (10% w/v) resulting in accordance with evidence reported in the present study (Randazzo et al. 2021). Differently, the salt resistance feature is not widespread in *L. plantarum* strains; in fact, our data revealed that only 1 strain out of 4 *L. plantarum* strains demonstrated 10% (w/v) NaCl tolerance, confirming data reported by Karasu et al. (2010) where only 2 out of 12 *L. plantarum* strains were able to grow at a salt concentration of 9% (w/v), suggesting the low tolerance of *L. plantarum* strains to high salt concentration. To tolerate high salt concentrations, LAB develops various strategies such as the uptake or the synthesis of a limited number of solutes (Júnior et al. 2015). The ability of NSLAB strains to grow in a wide range of salt concentrations is fundamental since they are often subjected to high concentrations of NaCl especially during brining and ripening of cheese production. In fact, NaCl is a common preservative for long-term storage cheeses and is crucial for managing cheese ripening (Georgieva et al. 2009).

Regarding the acidifying parameter generally, to be classified as a starter, a bacterial strain should be able to lower the pH of the milk

to 5.3 after 6 h (Beresford et al. 2001). According to that, all strains are often classified into three groups in line with their rate of acidification: fast acidification (pH < 5.3 after 6 h of fermentation), medium (pH < 5.3 after 8 h of fermentation), and slow (pH > 5.3 after 8 h of fermentation). As reported in **Figure 1**, the strains tested in the present study showed a Δ pH within 6 h ranging from 0.16 to 2.01, while the Δ pH within 8 h was from 0.22 to 2.08. According to that and as displayed by PCA plot (**Supplementary Figure S1**), *L. plantarum* strains (Q1C6 and Q3C4) demonstrated fast acidifying activity, 5 *L. delbruekii* stains (P14, P15, P36, P37, and P38) medium behavior, while all the others showed low acidifying activity proving to be usable for an adjunct culture. Similar results were obtained by Hadeif et al., (2022) demonstrating the weak acidification among the 36% LAB strains tested. Moreover, lactobacilli strains isolated from Tenerife cheese by Pérez et al. (2003) revealed low acidification activity. It is well known that LAB strains, selected as adjunct cultures, should have low acidifying activity; in fact, a high rate could generate sensory defects in cheese (Scatassa et al. 2015).

Table 2. Salt tolerance of LAB strains.

Isolates	2%	6%	10%
P14, P38, P50, Q3C4	+	+	+
P9, P10, P11, P13, P36, P37, P39, Q1C6, Q1C8, Q3C1, Q6C4, Q6C5, Q5C6	+	+	-
P7, P12, P15, P33, P40, Q3C3, Q5C9, Q6C1, Q22C2	+	-	-

(+), positive; (-), negative.

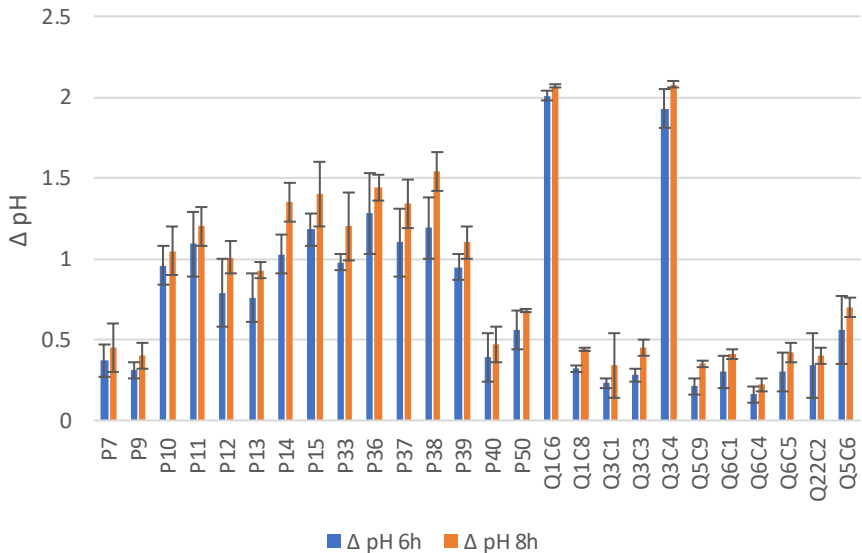


Figure 1. Acidifying activity of LAB strains. Results are expressed as Δ pH (6h and 8h) (starting from a pH of 6.7) and

reported as mean and standard deviation of three replicates.

3.4.3 *Aminopeptidase Activities*

In the present study, the aminopeptidase activities Pep N and Pep X were tested using Lys-pNa and H-Ala-Pro-pNa substrates, respectively. In detail, as reported in [Figure 2](#), the tested strains showed high activity against the substrate Lys-pNa, with values ranging from 265.47 to 2.15, of units per milligram of protein per minute (U/mg). In particular, according to the PCA plot ([Supplementary Figure S1](#)), the strain Q5C6 ascribed to the *L. lactis* species, as well as the *L. delbrueckii* P10 strain and the *L. rhamnosus* P50 strain were considered the best performers. Pep N data present in this study were higher to those obtained by Morea et al. (2007) in fact, *L. delbrueckii*, *L. gasseri* and *P. pentosaceus* strains isolated from Caciocavallo Pugliese cheese showed values ranging from 45.3 to 10.01 U/mg. Further, González and co-workers (2010) demonstrated low Pep N activity among LAB strains isolated from traditional Spanish cheese; in fact, except for few cases, the Pep N activity was generally low or even undetectable. Moreover, the *L. rhamnosus* P50 strain, tested in the present study, showed very high activity against the Lys-pNa substrate (254.56 U/mg) compared to *L. rhamnosus* strain tested by Carafa et al. (2015), which revealed an activity equal to 19 U/mg.

Overall, it is well known that aminopeptidase activity is crucial for the breakdown of peptides and the release of amino acids during the secondary proteolysis of the cheese. In particular, Pep N displays high selectivity for the basic amino acids Lys and Arg, followed by the hydrophobic/uncharged residues Leu and Ala (Christensen et al. 1999). It was previously isolated from strains ascribed to different

species such as *Lacticaseibacillus casei*, *Lactobacillus delbrueckii*, *Lactobacillus helveticus*, and *Streptococcus thermophilus* suggesting that this activity is strain-dependent and not species-dependent.

Concerning Pep X, the tested strains showed values ranging from 36.55 to 1.18 U/mg ([Figure 3](#)). Higher Pep X activity was displayed by the strain Q5C6 (33.22 U/mg), ascribed to the *L. lactis* species, as well as by the *L. delbrueckii* P10 (25.86) strain and by the *L. rhamnosus* P50 (36.55 U/mg) strain. In agreement with our results, some *L. rhamnosus* strains, isolated from semihard artisanal goat cheeses, showed a marked activity for Pep X when tested through Arg-Pro-pNA and Gly-Pro-pNA substrates (Meng et al. 2018), confirming that *L. rhamnosus* possesses a complex proteolytic system, including the X-prolyl-dipeptidyl aminopeptidase (Moslehishad et al. 2013). Differently Psoni et al. (2007), testing *L. lactis* strains isolated from Batzos cheese, revealed Pep X values lower than 5 U/mg. Similarly, Vlieg et al. (2006), testing a collection of dairy and wild *L. lactis* strains, obtained values of Pep X activity lower than 1 $\mu\text{mol}/\text{mg min}$. Pep X was purified and characterized from strains ascribed to the *L. acidophilus*, *L. casei*, *L. helveticus*, *L. lactis*, and *S. thermophilus* species (Christensen et al. 1999). The synergistic effect between Pep N and Pep X is essential to obtain high levels of hydrolysis. In fact, Pep N releases amino acids from the N-terminal of peptides and the rate of hydrolysis is reduced in presence of proline residues (Stressler et al. 2013). To compensate for the proline inhibition, the Pep X is able to release Xaa-Pro dipeptides from the N-terminal side of peptides. It is advantageous to have strains that can hydrolyze proline-containing peptides, since hydrophobic peptides with at least one proline residue have been linked to the bitter flavor of cheese (Habibi-Najafi et al. 1996).

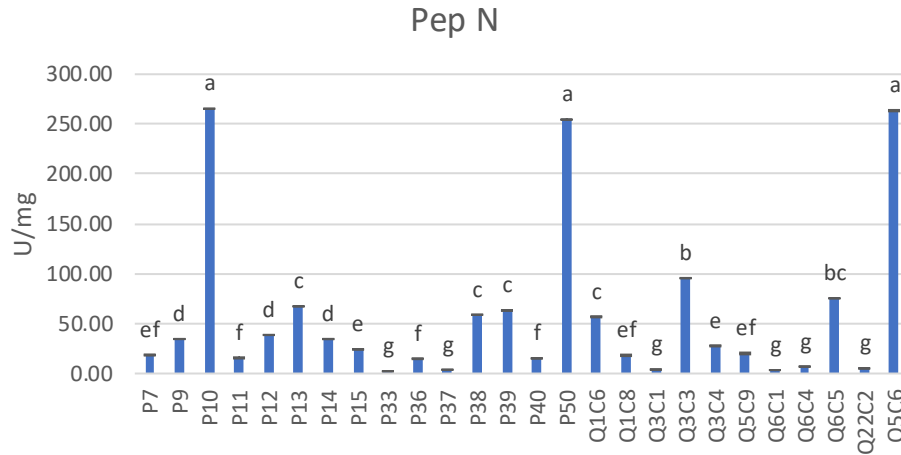


Figure 2. Aminopeptidase N (Pep N) activity expressed as mmol/min*mg proteins. Results are reported as mean and standard deviation of three replicates. Aminopeptidase activity was expressed as the number of activity units per milligram of protein per minute (U/mg). One unit of aminopeptidase activity was considered as the amount of enzyme required to release 1 μ mol of p-NA per minute under the assay conditions. Different letters (a-g) indicate statistically significant differences as determined by the one-way ANOVA test, which is followed by the Tukey's post-hoc test ($p < 0.05$).

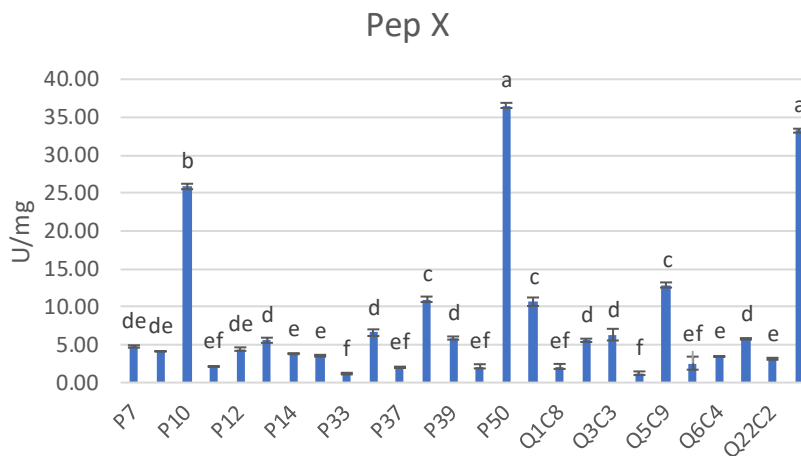
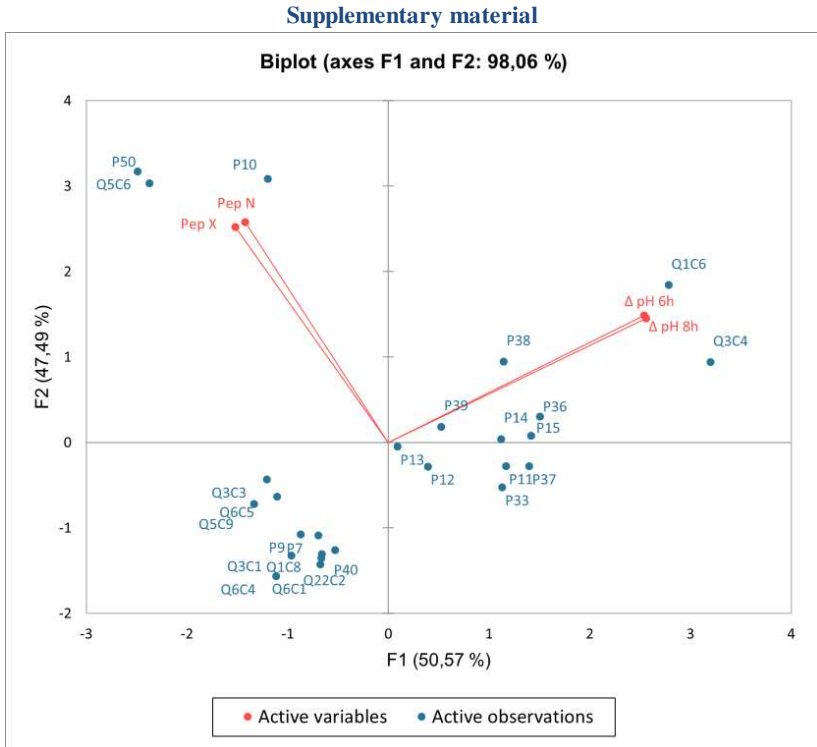


Figure 3. X-prolyl-dipeptidyl aminopeptidase (Pep X) activity expressed as mmol/min*mg pro-teins. Results are reported as mean and standard deviation of three replicates. Aminopeptidase activity was expressed as the number of activity units per milligram of protein per minute (U/mg). One unit of aminopeptidase activity was considered as the amount of enzyme required to release 1 μ mol of p-NA per minute under the assay conditions. Different letters (a-f) indicate statistically significant differences as determined by the one-way ANOVA test, which is followed by the Tukey's post-hoc test ($p < 0.05$).

3.5 Conclusions

In the present study, a technological characterization of indigenous LAB, isolated from Italian and Brazilian cheeses was carried out. Based on salt resistance, low acidifying and lipolytic activities, ability to produce diacetyl and EPS as well as aminopeptidase activities, data allowed us to select the strains Q5C6, P10, and P50, ascribed to *L. lactis*, *L. delbrueckii*, and *L. rhamnosus*, respectively, as the most promising to be used as adjunct cultures. In particular, the high aminopeptidase N and X activities displayed by the strains could help to enhance the flavor properties of cheese, improving the overall quality of the final product. In addition, the synergistic effect of aminopeptidase N and X revealed by the selected strains could be useful for the reduction of bitter peptides, generated from milk-clotting enzymes during cheese manufacture and ripening.

Research efforts should be made to confirm the results of the present study. The potential flavor improvement of ewe's and cow's milk cheese prepared using the selected strains will be further investigated at both pilot and industrial scales.



Supplementary Figure S1. Biplot of the principal components analysis (PCA) of LAB strains isolated from Brazilian and Italian cheeses in relation to aminopeptidase activities (Pep N and Pep X) and acidifying activity (Δ pH 6h and Δ pH 8h).

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Chapter 4



4 Chapter 4: *Lactococcus lactis* subsp. *lactis* bv. diacetyllactis Q5C6 strain as debittering adjunct culture for vegetable clotted cheese

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4.1 Abstract

One of the main challenges associated to the use of vegetable coagulants in cheesemaking is represented by the bitter taste conferred to the final product. In this context, the use of aminopeptidases positive strains could lead the hydrolysis of bitter peptides. The present study aimed to setup an experimental cheese clotted by using a kiwifruit enzymatic extract and inoculated with the *Lactococcus lactis* subsp. *lactis* bv. diacetylactis Q5C6 strain as debittering adjunct culture. The optimal amount of kiwifruit enzymatic extract, to be used in cheesemaking, was determined by performing laboratory-scale coagulation tests. Two experimental cheesemaking trials (A and B) were performed and the obtained cheeses were subjected to physico-chemical, microbiological, and sensory analysis. Results showed that, compared to cheeses clotted by animal rennet, the use of the kiwifruit enzymatic extract determined changes in fat, ash, and protein content. The *L. lactis* subsp. *lactis* bv. diacetylactis Q5C6 strain, inoculated at 1%, was able to reduce the bitter taste obtaining a final product with a sensory profile comparable to cheese clotted by animal rennet.

4.2 Introduction

Milk-clotting enzymes have a key role in cheesemaking, allowing the destabilization, and in turn the consequent aggregation of casein micelles forming the curd. Since ancient times, among milk-clotting enzymes, the animal rennet, obtained from the fourth stomach of suckling ruminants (such as calf), was widely used (Harboe et al. 2010). Nowadays, the use of conventional animal rennet in cheesemaking is limited due to many factors, such as religious restrictions (Islam and Judaism), dietary habits (such as vegetarianism), or economic factors. For these reasons, the attention of dairy companies was turned towards other milk clotting enzymes that are not of animal origin, such as microbial, recombinant, or vegetable extract (Nicosia et al. 2022a; Liu et al. 2021; Vaccalluzzo et al. 2020). Proteases of vegetable origin are traditionally used in cheesemaking in the Mediterranean, West African, and Southern European regions (Roseiro et al. 2003). Compared to chymosin, able to specifically hydrolyze the Phe₁₀₅-Met₁₀₆ k-casein bond, plant-derived proteases, with a low hydrolysis specificity for caseins, lead to the formation of some defects during cheese ripening (Mazorra-Manzano et al. 2013). In fact, the significant proteolytic activity of vegetable proteases, cause the formation of short peptides mainly conferring acidic and bitter taste (Oner and Akar, 1993). The latter is caused by the presence, in the side chains of peptides, of amino acids, such as lysine, leucine, and proline, which have high hydrophobicity values. According to Ney (1971), a peptide with a Q value higher or equal to 1400 certainly confers bitter taste. Differently, if hydrophobic amino acids are in a terminal position or are totally free, the Q value is below 1400 resulting in less perceived bitter taste. Among vegetable coagulant the Actinidin (EC. 3.4.22.14), a cysteine protease of

kiwifruit, with a molecular mass of 23.5 kDa and an optimal MCA/PA ratio, is considered a promising plant derived clotting enzyme (Lo Piero et al. 2011, Mazorra-Manzano et al. 2013, Nicosia et al. 2022b). However, the high proteolytic activity of plant derived enzymes, like kiwifruit extract, could lead to bitter taste in the final product.

Lactic acid bacteria possess complex systems of exopeptidases (aminopeptidases and carboxypeptidases) capable of hydrolyzing milk caseins to satisfy their needs in amino acids and small peptides able to generate pleasant flavour compounds especially in traditional cheeses (Randazzo et al. 2008, 2010; Caggia et al. 2015; Guarcello et al. 2016; Carpino et al. 2017; Pino et al. 2018). These exopeptidases can be used to debitter protein hydrolysates reducing the Q values of peptides (Raksakulthai and Haard 2003). Among exopeptidases, both the aminopeptidase N (PepN; EC. 3.4.11.2) and the X-Prolyl-dipeptidyl peptidase (PepX; EC 3.4.14.11), which act synergistically, are involved in the debittering process. In detail, the PepN is capable of hydrolyzing hydrophobic amino acids from the N-terminal of peptides with a reduced rate of hydrolysis in the presence of proline (Stressler et al. 2013). Differently, the X-prolyl dipeptidase (PepX) has a high specificity of hydrolysis for peptides that contain proline, thus compensating for the lack of Pep N (Habibi-Najafi and Lee, 1996). Both PepN and PepX activities were extensively studied in lactic acid bacteria of food origin as previously reported by Nicosia and co-workers (2023). According to the study, although, *Lactococcus lactis* is generally used as a starter culture in cheesemaking, the *Lactococcus lactis* subsp. *lactis* bv. diacetylactis Q5C6 strain showed marked PepX and PepN activities, proving to be a good candidate as debittering adjunct culture (Nicosia et al. 2023).

The aim of the present work was to setup an experimental cheese clotted by using a kiwifruit enzymatic extract and inoculated with the *L. lactis* subsp. *lactis* bv. diacetylactis Q5C6 strain as

debittering adjunct culture.

4.3 Material and methods

4.3.1 Preparation of the kiwifruit enzymatic extract

The kiwifruit enzymatic extract (KEE) was obtained from *Actinidia deliciosa* cv. Hayward fruits purchased in a local market in Viçosa (Minas Gerais, Brazil). In detail, kiwifruits were pressed using a hydraulic press (ABF Technologies, Cormeilles) and, after centrifugation at 8500 rpm for 20 minutes, the obtained kiwifruit aqueous extract was subjected to salting out technique as follow: kiwifruit aqueous extract was saturated at 45% through the gradual addition of NaCl (w/v), then centrifugated at 9000 rpm for 20 min at 4 °C. After discarding the supernatant, the resulting precipitate was used as KEE in cheesemaking tests.

4.3.2 Laboratory-scale coagulation tests

Laboratory-scale coagulation tests were performed to evaluate the coagulation features of the KEE. In detail, whole cow milk was heated at 37 °C and inoculated with different concentrations of the KEE (3, 1.5, 0.7, 0.25 g/L). The milk-clotting activity of the KEE was performed as reported by Benheddi and Hellal (2019).

4.3.3 Experimental cheesemaking

Two different experimental cheesemaking trials (A and B) were carried out using whole cow milk (three repetitions of each trial were conducted at different days) at the Laboratório de Pesquisa em Leites e Derivados (InovaLeite, Federal University of Viçosa, Minas Gerais, Brazil). Delvo Cheese CT-111 (DSM Food Specialists, the Netherlands) was used as starter, whereas the *L. lactis* subsp. *lactis* bv. diacetylactis Q5C6 strain, previously isolated from Pará cheese (Martins et al. 2020; Fusieger et al. 2020) and characterized for aminopeptidase activity (Nicosia et al. 2023) was used as debittering adjunct culture. Q5C6 strain was revitalized twice in reconstituted skim milk ($10 \text{ g} \times 100 \text{ mL}^{-1}$ autoclaved for 10 min at $115 \text{ }^\circ\text{C}$) and incubated at $37 \text{ }^\circ\text{C}$ for 24 h, then used at final cell density of $10^8 \text{ CFU} \times \text{mL}^{-1}$ in cheesemaking.

Fig. 1 shows the flowchart of the experimental cheesemaking trial A. In detail, the milk (30 L) was heat treated at $65 \text{ }^\circ\text{C}$ for 30 minutes, cooled to $37 \text{ }^\circ\text{C}$ then inoculated with the starter culture (1% v/v). Three fermentation batches (AKD, AK, and AC), each one consisting of 10 L of milk, were setup. In detail, the AKD and AK samples, with and without the addition of the Q5C6 debittering strain, were clotted using the KEE (0.7 g/L) and maintained at $37 \text{ }^\circ\text{C}$ for 90 min. The AC sample, without the inoculum of the Q5C6 debittering strain, was clotted using commercial rennet (0.05 mL/L) at $37 \text{ }^\circ\text{C}$ for 40 min and used as control sample. After incubation, the curd, obtained from each fermentation batch, was cut into small granules (4–6 mm), then transferred into cheese molds and pressed to facilitate the purging of the whey. Samples were immersed in brine (15% w/v) then stored under controlled conditions of temperature and humidity (15°C , 90-92% RH).

The flowchart of the experimental cheesemaking B is reported in Fig. 2. The milk (40 L) was pretreated as previously reported and, after starter culture addition, four fermentation batches (BKD1,

BKD2, BKD3, and BC), each one consisting of 10 L of milk, were setup. The BKD1, BKD2, and BKD3 samples were clotted using the KEE (0.7 g/L) and inoculated with 1%, 2%, and 3% (v/v) of the Q5C6 debittering strain, respectively. After 90 minutes at 37 °C, the curd was processed as previously described. The BC sample, used as control, was obtained following the same procedure applied for the production of the AC sample, as described above.

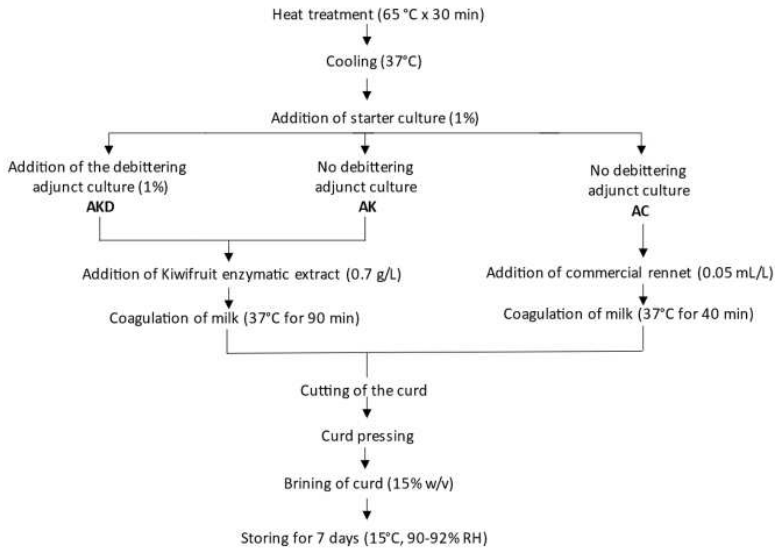


Fig. 1. Flow chart of the experimental cheesemaking A using the KEE (kiwifruit enzymatic extract) and the *L. lactis* subsp. *lactis* bv. *diacetylactis* Q5C6 strain as debittering strain. AKD, clotted with KEE and with the addition of the Q5C6 debittering strain; AK, clotted with KEE and without the addition of the Q5C6 debittering strain; AC, clotted using commercial rennet and without the addition of the Q5C6 debittering strain.

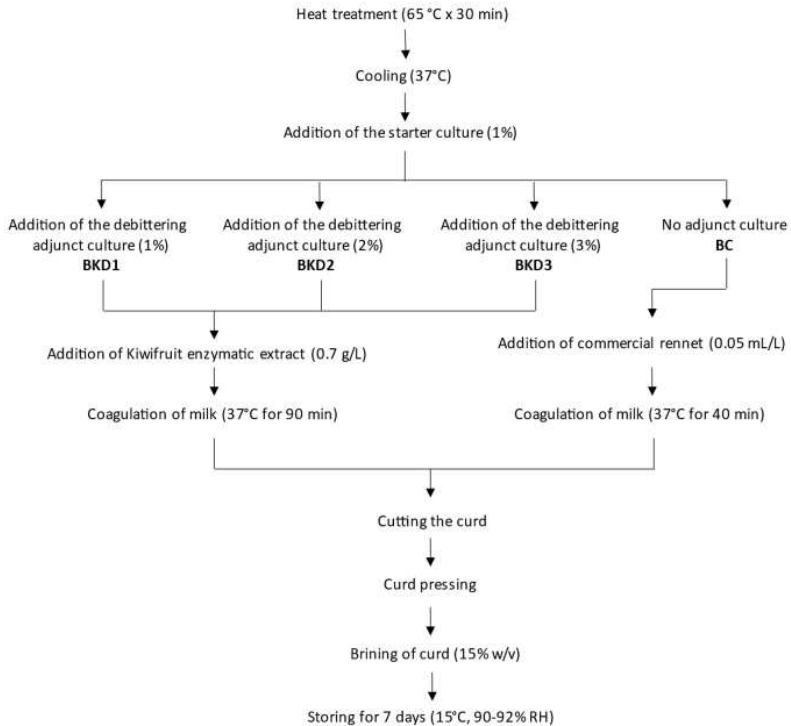


Fig. 2. Flow chart of experimental cheesemaking B using the KEE (kiwifruit enzymatic extract) and different concentrations of the *L. lactis* subsp. *lactis* bv. *diacetylactis* Q5C6 strain used as debittering strain. BKD1, cheese clotted using KEE with the addition of the Q5C6 debittering strain (1%); BKD2, cheese clotted using KEE with the addition of the Q5C6 debittering strain (2%); BKD3 cheese clotted using KEE with the addition of the Q5C6 debittering strain (3%); BC, clotted using commercial rennet without the addition of the Q5C6 debittering strain.

4.3.4 Physico chemical analysis

Physico chemical analyses of cheese samples were performed after 7 days of storage at 15 ± 1 °C. The moisture content was determined gravimetrically by drying 5 g of samples at 105 ± 2 °C until a constant mass was obtained (ISO 5534:2004). Fat content was measured by Gerber-van Gulik method (ISO 3432:2008). Protein was calculated by determination of total nitrogen by the Kjeldahl method, using a conversion factor of 6.38 (ISO 8968-1:2014). The total content of ash was determined gravimetrically by the incineration method at 550°C (IDF 27:1964). For pH, 10 g of sample were homogenized in 40 mL of distilled water. After 5 minutes, the mixture was filtered through cotton wool and the pH was measured (at a temperature of 25 ± 1 °C) using a pH meter (Kasvi K39-1014B, São José dos Pinhais, PR, Brazil) (Pino et al. 2021; Randazzo et al. 2021). Each analysis was performed in triplicate and results are reported as mean and standard deviation.

4.3.5 Microbiological analysis

One-week old cheese samples were subjected to culture-dependent analysis. In detail, 25 g of sample were diluted using 225 ml of NaCl solution (0.90% w/v) and homogenized using a stomacher (BagMixer® 400, Interscience, Saint Nom, France) for 90 seconds. Decimal dilutions were obtained and plated using the following agar media and conditions: Plate Count Agar (PCA) (Kasvi, São José dos Pinhais, PR, Brazil), aerobically incubated at 30 °C for 48–72 h, for total mesophilic count; MacConkey agar (Merck, Darmstadt, Germany) anaerobically incubated at 36 °C for 18 h for Enterobacteria

enumeration; M17 agar (HiMedia, Mumbai, MH, India), incubated at 30 °C for 24–48 h, for lactococci count; Potato Dextrose Agar (K25-1022, Kasvi) supplemented with 1.5% of tartaric acid solution (10% w/v) incubated at 25 °C for 3–5 days for yeasts; Mannitol Salt Agar (MSA) (Kasvi), incubated at 32 °C for 48 h for staphylococci detection; de Man Rogosa and Sharpe agar (MRS), incubated at 32 °C for 72 h, under microaerophilic condition, for lactobacilli (Kasvi) (Randazzo et al. 2009). The presence of *Listeria monocytogenes* and *Salmonella* spp. was evaluated following the method previously described by Pino et al. (2018) and Carpino et al. (2017). Results are expressed as mean log cfu/g and standard deviation of three replicates.

4.3.6 Sensory analysis

4.3.6.1 Ranking test

The AKD, AK, and AC samples obtained from the experimental cheesemaking trial A, were subjected to ranking test for bitter taste. Forty (40) untrained panelists, 17 male and 23 female, aged between 18 and 51 years, participated to the sensory analysis. The test was conducted in individual booths, under white light, at the UFV's Technological Innovation Laboratory (Viçosa, Minas Gerais, Brazil). Cheese samples (approximately 10 g), at a temperature of 10°C, were presented in black disposable plastic plates, coded with three random digits and served with toothpicks. Panelists were asked to taste randomized cheese samples, from left to right, and to score them as slightly (score 1), medium (score 2) or very (score 3) bitter.

Results, representing the sum of the obtained scores, were evaluated according to the Friedman test ($p < 0.05$). The research protocol followed the guidelines of the Helsinki declaration and all procedures involving human subjects were approved by the Committee on Ethics in Human Beings Research of the Universidade

Federal de Viçosa (n° 3.516.953).

4.3.6.2 RATA test

The RATA test (Rate-all-that-apply) (Meyners, Jaeger and Ares, 2016) was applied to define the sensory profile of the cheese samples obtained from the experimental cheesemaking trial B (BKD1, BKD2, BKD3, and BC). Seventy (70) panelists (57% female, 43% male, ages: 18 to 62) were recruited among staff, students and visitors of the Federal University of Viçosa (Viçosa, MG, Brazil). Cheese samples (10 g at a temperature of 10°C) were presented in black disposable plastic plates, coded with three random digits and served with toothpicks. Panelists were asked to evaluate the following descriptors: firmness, gumminess, ricotta consistency, pasty, cream cheese texture, brittleness, grittiness, creaminess, vegetable taste, milk taste, rancidity, astringent, fruity, bitterness, acid taste, salty taste, spicy, animal odor, biscuit odor, stickiness, moisture, and shiny appearance using a 6-point scale ranging from 0 (not applicable) to 5 (extremely applicable). In addition, panelists were asked to rate, through a 9-point scale, the overall acceptability. For each descriptor, the score attributed to each sample were subjected to one-way ANOVA followed by a pairwise multiple comparison test (Tukey HSD). Differences were considered statistically significant at $p < 0.05$. In addition, RATA test score was subjected to Principal Component Analysis (PCA). The research protocol followed the guidelines of the Helsinki declaration and all procedures involving human subjects were approved by the Committee on Ethics in Human Beings Research of the Universidade Federal de Viçosa (n° 3.516.953).

4.3.7 Statistical analysis

One-way ANOVA followed by Tukey post-hoc test ($p < 0.05$) was applied to physico-chemical and microbiological data using the XLSTAT® software (version 2022.4.1.1365, Addinsoft, New York) and to RATA test data using the Minitab 16.0 software (Minitab Inc., State College, PA, USA). Principal Component Analysis (PCA) was performed using the XLSTAT® software (version 2022.4.1.1365, Addinsoft, New York).

4.4 Results

4.4.1 Laboratory-scale coagulation tests

Table 1 shows the coagulation abilities of different concentration of the KEE (3, 1.5, 0.7 and 0.25 g/L) as well as the perception of the bitter taste. Among the tested concentration of the KEE, 0.07 g/L was selected allowing to obtain low bitter taste and acceptable clotting time.

Table 1. Coagulation features of different KEE concentrations and perception of the bitter taste

Concentration of kiwifruit extract in milk (g/L)	Observations
3	Coagulation occurred very quickly (10 min). The increased proteolytic activity led to the development of an extremely strong bitter taste.
1.5	Coagulation time comparable to commercial rennet (50 min). Pronounced bitter taste
0.7	Slow coagulation (90 min) resulting in a firm clot. Slight aftertaste of bitterness
0.25	Very slow coagulation (160 min) resulting in a soft clot. Imperceptible bitter taste

4.4.2 *Physico-chemical analysis of experimental cheeses*

Tables 2 and 3 report moisture, fat content, ash, protein, and pH values of cheese samples obtained from the experimental cheesemaking trial A and B, respectively. Concerning cheese samples obtained from the experimental cheesemaking trial A, no statistically significant differences were recorded among samples for moisture, ash, and pH. The fat content was higher in cheeses coagulated with the KEE (AKD and AK) than cheese obtained using commercial rennet (AC). Regarding to protein content, AC and AK samples showed similar values whereas a low concentration of protein (16.36%) was registered in AKD sample (Table 2). Among cheese samples obtained from the experimental cheesemaking trial B, significant differences were observed for ash, protein, and pH (Table 3). Overall, samples obtained using the KEE (BKD1, BKD2, and BKD3) showed lower values, for all the parameters, than those observed in BC sample. In detail, the ash content of cheese coagulated with KEE ranging from 2.70 to 2.88% while the BC sample show statistical difference content with 3.20%. As regards the protein content, samples BKD1, BKD2 and BKD3 (21.54, 21.36 and 21.18% respectively) show statistical differences with sample BC which showed a value of 23.40%. Finally, the pH of the experimental samples was around 4 while the highest was recorded in the BC sample (5.28).

Table 2. Moisture, fat, ash, protein, and pH of cheese samples obtained from the experimental cheesemaking trial A.

	AC	AKD	AK
Moisture %	51.89 ± 0.14 ^a	49.9 ± 0.20 ^a	48.5 ± 0.23 ^a
Fat %	24.54 ± 0.20 ^b	30.51 ± 0.27 ^a	29.2 ± 0.01 ^a
Ash %	2.43 ± 0.03 ^a	2.41 ± 0.01 ^a	2.22 ± 0.04 ^a
Protein %	20.16 ± 0.20 ^a	16.36 ± 0.11 ^b	19.41 ± 0.19 ^a
pH	5.85 ± 0.02 ^a	5.46 ± 0.05 ^a	5.77 ± 0.05 ^a

Data are reported as average values and standard deviations of three replicates.

AC, clotted using commercial rennet and without the addition of the Q5C6 debittering strain; AKD, clotted with KEE and with the addition of the Q5C6 debittering strain; AK, clotted with KEE and without the addition of the Q5C6 debittering strain.

Table 3. Moisture, fat, ash, protein, and pH of cheese samples obtained from the experimental cheesemaking trial B.

	BC	BKD1	BKD2	BKD3
Moisture %	47.18 ± 0.08 ^a	46.06 ± 0.03 ^a	45.72 ± 0.07 ^a	46.23 ± 0.11 ^a
Fat %	25.30 ± 0.05 ^b	29.05 ± 0.00 ^a	29.55 ± 0.00 ^a	29.00 ± 0.00 ^a
Ash %	3.20 ± 0.10 ^a	2.88 ± 0.06 ^b	2.70 ± 0.04 ^b	2.86 ± 0.02 ^b
Protein %	23.40 ± 0.13 ^a	21.54 ± 0.20 ^b	21.36 ± 0.16 ^b	21.18 ± 0.07 ^b
pH	5.28 ± 0.09 ^a	5.16 ± 0.06 ^a	4.08 ± 0.01 ^b	4.11 ± 0.05 ^b

Data are reported as average values and standard deviations of three replicates.

BC, clotted using commercial rennet without the addition of the Q5C6 debittering strain; BKD1, cheese clotted using KEE with the addition of the Q5C6 debittering strain (1%); BKD2, cheese clotted using KEE with the addition of the Q5C6 debittering strain (2%); BKD3 cheese clotted using KEE with the addition of the Q5C6 debittering strain (3%).

4.4.3 Microbiological analysis of experimental cheeses

Tables 4 and 5 show the mean values and standard deviations of the main microbial groups detected in cheese samples obtained from experimental cheesemaking trial A and B, respectively. Overall, *Listeria* and *Salmonella* were never detected in the analyzed samples. No statistically significant differences were recorded among cheeses obtained from the experimental cheesemaking trial A, for mesophilic bacteria, lactobacilli, *Enterobacteriaceae*, and staphylococci. The ADK sample showed the highest cell density of lactococci (7.56 ± 0.07 log cfu/g) whereas the highest count of yeasts was observed in the AC sample (3.31 ± 0.04 log cfu/g) (Table 4). Concerning cheese samples obtained from the experimental cheesemaking trial B, no differences were observed in lactobacilli and *Enterobacteriaceae* cell densities. The lowest count of mesophilic bacteria was detected in BKD3 and BC samples, respectively. In addition, the BC sample showed the higher yeasts and staphylococci cell density.

Table 4. Microbial counts expressed as log cfu/g and standard deviation of the main microbial groups detected on cheese samples obtained from the experimental cheesemaking trial A.

	AC	AKD	AK
Mesophilic bacteria	7.72 ± 0.12 ^a	7.13 ± 0.08 ^a	7.22 ± 0.06 ^a
Lactobacilli	7.30 ± 0.11 ^a	7.43 ± 0.07 ^a	7.34 ± 0.08 ^a
Enterobacteriaceae	2.78 ± 0.05 ^a	2.51 ± 0.10 ^a	2.65 ± 0.12 ^a
Lactococci	6.53 ± 0.11 ^b	7.56 ± 0.07 ^a	6.85 ± 0.18 ^b
Yeasts	3.31 ± 0.04 ^a	2.56 ± 0.05 ^b	2.49 ± 0.16 ^b
Staphylococci	2.34 ± 0.08 ^a	2.20 ± 0.04 ^a	2.12 ± 0.05 ^a
<i>Listeria</i> spp.	<1	<1	<1
<i>Salmonella</i> spp.	<1	<1	<1

Data are reported as average values and standard deviations of three replicates.

AC, clotted using commercial rennet and without the addition of the Q5C6 debittering strain; AKD, clotted with KEE and with the addition of the Q5C6 debittering strain; AK, clotted with KEE and without the ad-dition of the Q5C6 debittering strain.

Table 5. Microbial counts expressed as log cfu/g and standard deviation of the main microbial groups detected on cheese samples obtained from the experimental cheesemaking trial B.

	BC	BKD1	BKD2	BKD3
Mesophilic bacteria	7.36 ± 0.06 ^a	7.65 ± 0.02 ^a	7.53 ± 0.06 ^a	6.54 ± 0.05 ^b
Lactobacilli	7.04 ± 0.08 ^a	7.51 ± 0.12 ^a	7.43 ± 0.07 ^a	7.81 ± 0.09 ^a
Enterobacteriaceae	2.72 ± 0.12 ^a	2.73 ± 0.06 ^a	2.86 ± 0.10 ^a	2.49 ± 0.03 ^a
Lactococci	6.45 ± 0.18 ^b	7.23 ± 0.11 ^a	7.38 ± 0.12 ^a	7.76 ± 0.02 ^a
Yeasts	3.79 ± 0.06 ^a	2.71 ± 0.04 ^b	2.56 ± 0.15 ^b	2.32 ± 0.02 ^b
Staphylococci	3.04 ± 0.05 ^a	2.34 ± 0.08 ^b	2.15 ± 0.04 ^b	2.64 ± 0.12 ^b
<i>Listeria</i> spp.	<1	<1	<1	<1
<i>Salmonella</i> spp.	<1	<1	<1	<1

Data are reported as average values and standard deviations of three replicates.

BC, clotted using commercial rennet without the addition of the Q5C6 debittering strain; BKD1, cheese clotted using KEE with the addition of the Q5C6 debittering strain (1%); BKD2, cheese clotted using KEE with the addition of the Q5C6 debittering strain (2%); BKD3 cheese clotted using KEE with the addition of the Q5C6 debittering strain (3%).

4.4.4 *Sensory analysis: ranking test*

Results of the ranking test, applied to cheese samples obtained from the experimental cheesemaking trial A are reported in Table 6. Overall, all samples (AC, ADK, and AK) were differently scored by panelists ($p < 0.05$). In detail, the bitter taste was considered low in the AC sample (score of 42) whereas the ADK and AK samples were categorized as averagely and highly bitter, respectively (Table 6).

Table 6. Sum of the ranking test scores.

n = 40	Samples		
	AC	AKD	AK
Rank sum	42 ^a	86 ^b	112 ^c

^{a-c}Different lowercase letters indicate statistically significant differences ($p < 0.05$) among samples according to Friedman's test. n, number of panelists. AC, clotted using commercial rennet and without the addition of the Q5C6 debittering strain; AKD, clotted with KEE and with the addition of the Q5C6 debittering strain; AK, clotted with KEE and without the addition of the Q5C6 debittering strain.

4.4.5 *Sensory evaluation: RATA test*

Fig. 3 shows the radar chart of the RATA test applied to cheese samples obtained from the experimental cheesemaking trial B (BC, BKD1, BKD2, and BKD3). Overall, out of the 22 selected descriptors, 13 (firmness, gumminess, ricotta consistency, pasty, cream cheese

texture, brittleness, creaminess, rancidity, astringent, bitterness, acid taste, stickiness, and shiny appearance) were statistically significant different among samples ($p < 0.05$). In particular, firmness (mean score of 4.21), gumminess (mean score of 3.34), and shiny appearance (mean score of 2.31) were mainly perceived in the BC sample; the BKD1 sample received the highest score for both ricotta consistency (mean score of 2.63) and brittleness (mean score of 1.78); pasty (mean score 2.56), cream cheese texture (mean score 1.73), creaminess (mean score 3.20), rancidity (mean score 1.17), astringent (mean score 1.63), bitterness (mean score 2.27), acid taste (mean score 3.07), and stickiness (mean score 2.06) were mainly perceived in BKD2 sample.

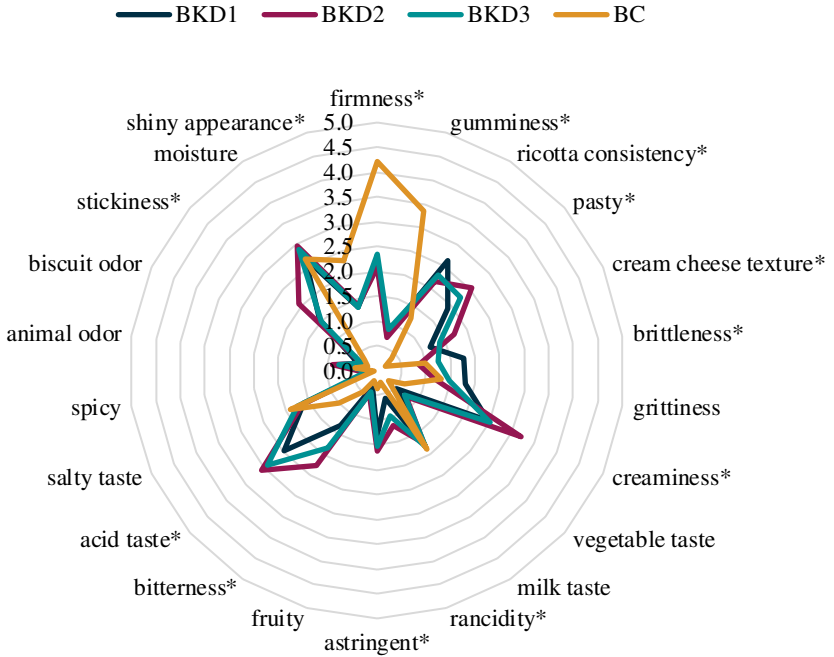


Fig. 3. Radar chart of the RATA test applied to cheese samples obtained from the experimental cheesemaking trail B (BC, BKD1, BKD2, and BKD3). Each descriptor was scored based on a 6 point scale (0, not applicable; 5, extremely applicable). * Statistical differences among samples at $p < 0.05$.

4.4.6 *Overall appreciation*

Fig. 4 shows the results of the 9-point scale overall appreciation. No statistically significant differences were detected by panelist between the control sample (BC, score of 6.629) and the cheese clotted using KEE with the addition of the Q5C6 debittering strain (1%) (BKD1, score of 5.914). Lower appreciation scores were attributed to BKD2 and BKD3 samples. Fig. 5 shows the Principal Component

Analysis (PCA) of the sensory profile of experimental cheese samples. PC1 and PC2 account for 99.43% variances in sensory descriptors showing that BC is associated with several descriptors, such as: gumminess, shiny appearance and firmness while the cheeses coagulated with vegetable enzyme were located at the opposite side along the first dimension, showing correlation with pasty, creaminess and acid taste attributes. Moreover, BKD2 and BKD3 samples cluster in the same quadrant of the graph associating themselves with attributes such as acid taste, bitterness and creaminess, and BKD1 sample differs from the other samples and from those attributes considered as "negative" by panelists.

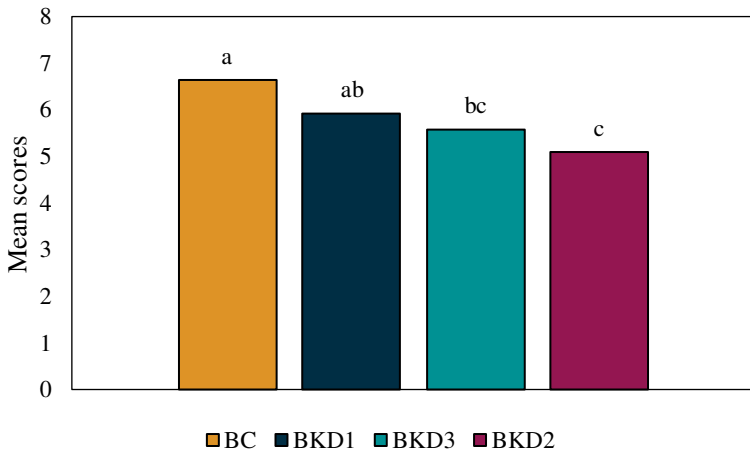


Fig. 4. Mean scores obtained by the experimental cheeses (BKD1, BKD2, BKD3 and BC) in the acceptance test (on a scale from 0 to 8, with 0 = “extremely disliked” to 8 = “extremely liked”) according to the inoculum percentage of debittering *L. lactis* subsp. *lactis* bv. *diacetylactis* Q5C6 strain. Statistical differences among samples at $p < 0.05$.

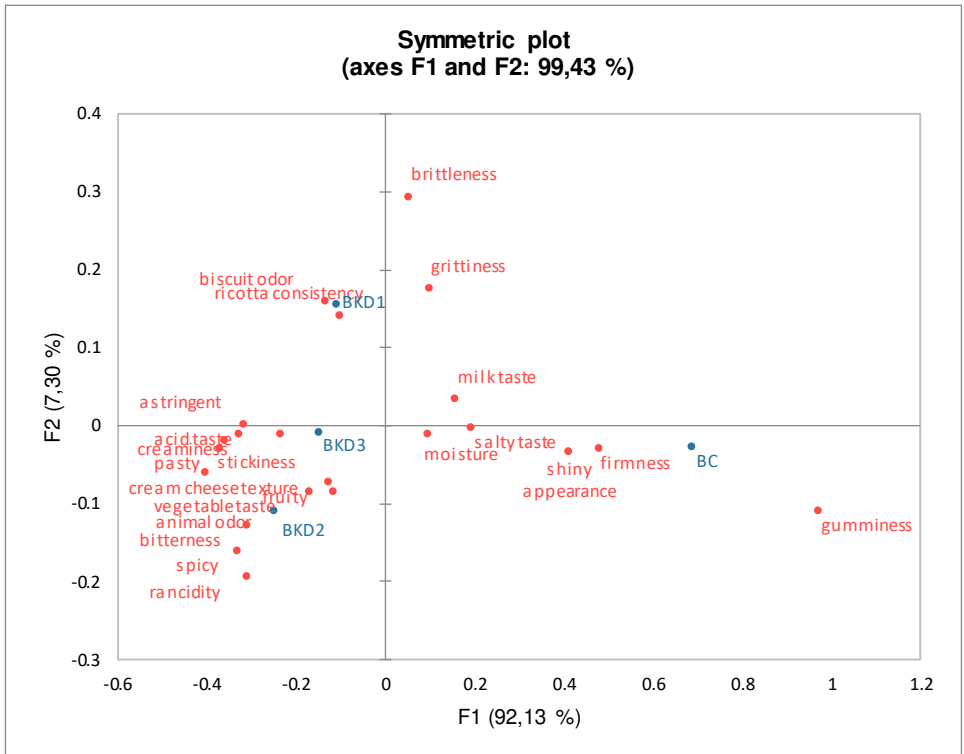


Fig. 5. Symmetric plot with dimensions 1 and 2 of Principal Component Analysis correspondence analysis (CA) of RATA (Rate-All-That-Apply) data from the sensory profiling of experimental cheeses (BKD1, BKD2, BKD3 and BC). The PCA was performed on the sum of scores given by all consumers to each term for describing each sample (RATA scoring).

4.5 Discussion

The global increase in cheese demand and the constant decrease in calf rennet supply, along with vegetarianism, religious restrictions (kosher and halal), and unfavorable consumer perceptions on the use of genetically modified microorganisms, contributed to increase the interest of dairy industries to use vegetal coagulants for cheese production (Jacob et al. 2011; Bathmanathan et al. 2019). Although some traditional cheeses in the Mediterranean area, Southern Europe, and West Africa are made using plant rennet, the main challenge to use vegetable coagulants is represented by the bitter taste conferred to the final product (Ben Amira et al. 2017). According to that, the present study aimed to setup an experimental cheese clotted by using a kiwifruit enzymatic extract and inoculated with the *L. lactis* subsp. *lactis* bv. *diacetylactis* Q5C6 strain as debittering adjunct culture.

It is well known that the enzymatic extract of kiwifruit can be considered a promising substitute for chymosin due to the ability to form a good milk clot (Nicosia et al. 2022b). In this context, an optimal concentration of kiwifruit extract must be chosen in order to shorten the coagulation time and reduce the bitter taste caused by the secondary proteolysis carried out by the residual enzyme. To obtain this goal, in the present study, the use of 0.7 g/L of the kiwifruit enzymatic extract allowed to obtain acceptable coagulation time and slight bitter taste. A similar approach was applied by Benheddi and Hellal (2019) using the crude enzymatic extract of *Cynara cardunculus*. In particular, by testing different concentrations of the aforementioned extract, the percentage of 0.5 (v/v) was considered appropriate to obtain a cheese with acceptable sensory properties without affecting the coagulation process.

As previously reported, the use of vegetable coagulants could

affect the physicochemical profile of the final product (Mazorra-Manzano et al. 2013). In particular, as observed in the present study, the experimental cheeses obtained using the kiwifruit enzymatic extract showed higher fat content than cheeses clotted using commercial rennet. The increase in fat content can be due to the greater ability of vegetable coagulant to retain fatty components in the curd. This finding agrees with Nuñez et al. (1991) who evaluated the chemical, rheological, microbiological, and sensory characteristics of the La Serena cheese obtained using a vegetable rennet from *Cynara cardunculus L.* as coagulant. Similarly, Sanjuán and co-workers (2002), by studying the effect of an aqueous extract of the thistle of *Cynara cardunculus L.* on physical and chemical characteristics of Los Pedroches cheese, highlighted a higher fat content in vegetable clotted cheeses compared to samples obtained using animal rennet as coagulant. Different results were obtained using apple leaves of *Calotropis procera* (Aworth and Muller 1987) or ash gourd proteinase (Gupta and Eskin 1977).

Concerning ash, which includes, along with salt added to the curd, natural mineral components of the milk, higher values were observed in vegetable clotted samples than in control ones. This observation is in line with data obtained by Sanjuán and co-workers (2001) in Los Pedroches cheese clotted by *Cynara cardunculus L.* extract.

Interestingly, the low protein content observed in cheese samples clotted by using the kiwifruit extract and inoculated with the debittering adjunct culture could be related to the proteolytic activity exerted by the *L. lactis* subsp. *lactis* bv. diacetylactis Q5C6 strain, as previously reported by Nicosia and co-workers (2023).

The use of aminopeptidase positive strains to counteract the bitter taste is well documented (Christensen et al. 1999). In this context, Habibi and Lee (2007) used a X-prolyl dipeptidyl peptidase,

extracted from the *Lactobacillus casei* spp. *casei* LLG strain, to reduce the bitterness in cheddar slurries supplemented with Neutrase® 0.5 L (neutral protease derived from *Bacillus subtilis*). The fast debittering, reported by the authors, was explained based on the ability of the enzyme to liberate an amino acid residue, containing proline at penultimate N-terminal position, from peptides with a Q value higher than 1400 (Habibi and Lee, 2007). Similarly, Tan et al. (1993) used the aminopeptidase N, extracted from the *Lactococcus lactis* subsp. *cremoris* WG2 strain, to debitter a complex peptide mixture of a tryptic digest from bovine beta-casein. Based on the obtained results, the ability of the studied aminopeptidases to reduce bitterness, by specific hydrolysis of high Q-value peptides, released from caseins, was demonstrated (Tan et al. 1993). Based on our knowledge, no previous studies were conducted to evaluate the ability of aminopeptidase positive strains to reduce the bitter taste in cheese clotted using vegetable enzymes. According to that, in the present study, the use of the *L. lactis* subsp. *lactis* bv. *diacetylactis* Q5C6 strain, able to exert both Pep N and Pep X aminopeptidase activities, determined a significant reduction of the perceived bitter taste by panelists. However, further studies will need to clarify the viability of Q5C6 strain over time as well as the rate of autolysis, in order to detect when cell lysis, releasing aminopeptidases into the matrix. Furthermore, GC analysis will be carried out to determine which molecules are responsible of sensorial differences in cheeses at different ripening times. In addition, as demonstrated by sensory data, the percentage of the inoculated debittering adjunct culture significantly influenced both the sensory profile and the overall acceptability of the final product. More in depth, increased concentrations of the adjunct culture were associated to higher perception of descriptors usually associated to cheese defects (e.g. rancidity, acid taste, creaminess, stickiness, etc), determining in turn

the reduction of the overall appreciation of the final product. However, although *L. lactis* subsp. *lactis* bv. diacetylactis species produces high amount of acetoin and diacetyl related to buttery flavour notes to dairy products, this aspect was not evaluated, representing a weakness of the present study.

4.6 Conclusions

In conclusion, the present work confirmed the in vivo debittering activity of the *L. lactis* subsp. *lactis* bv. diacetylactis Q5C6 strain through cheesemaking trials. Moreover, results demonstrated that a combination of 0.7 g/L of kiwifruit enzymatic extract with 1% of the debittering *L. lactis* subsp. *lactis* bv. diacetylactis Q5C6 strain resulted in an appreciated cheese with overall acceptability comparable to those obtained using commercial rennet. Further studies will be conducted in order to evaluate the effect of the debittering adjunct culture on proteolysis and sensory profiles of vegetable coagulated cheeses produced at industrial scale and with different ripening times.

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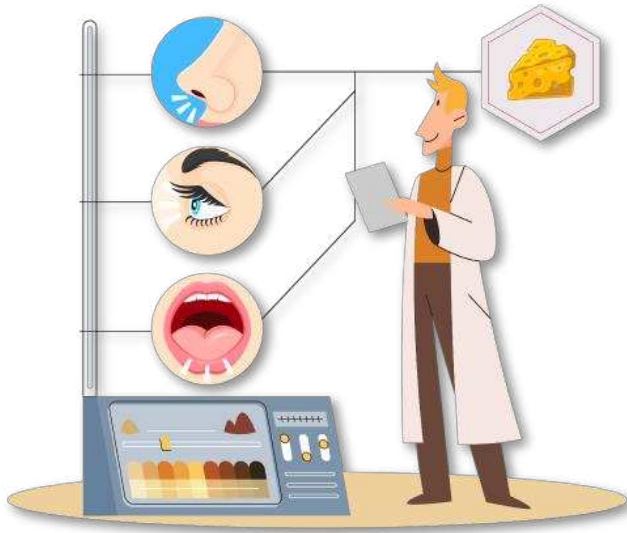
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Chapter 5



5 Chapter 5: Artificial sensory evaluation and microbiological assessment of Sicilian cheese made with plant-derived extract coagulant

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5.1 Abstract

In the present study, cheeses coagulated with different milk-clotting enzymes were investigated. In detail, commercial animal rennet, microbial coagulant, and kiwifruit enzymatic extract were used in the cheesemaking process, and the different cheeses were examined. In detail, microbiological and chemical analysis were carried out and the organoleptic profiles were assessed analytically through E-Eye, E-Nose and E-Tongue platforms. Results obtained from the microbiological analysis showed higher presence of lactobacilli and lactococci in cheeses clotted with animal rennet and vegetable coagulant. This data is correlated to the higher presence in these samples of carboxylic acids, aldehydes, alcohols, and other compounds derived from the metabolism of the LAB, that are important components with a key role in the flavor development of cheese. These data were confirmed by artificial senses analysis, showing that cheese coagulated with vegetable enzyme exhibited organoleptic profile comparable to the cheese coagulated with traditional animal rennet. Additionally, the vegetable clotted cheese revealed higher phytosterols and polyphenols compounds as well as a lower presence of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) allowing to obtain a final product with healthy and safety properties.

5.2 Introduction

Calf rennet, obtained from the abomasum of suckling calves, has been used for milk coagulation in cheesemaking for centuries (Lopes et al., 1998). The milk-clotting feature of calf rennet is due to the presence of chymosin: an aspartic protease that specifically cleave the Phe₁₀₅-Met₁₀₆ peptide bond of κ -casein, initiating the coagulation process of caseins. Nowadays, due to the global increase in cheese production and consumption, calf rennet is sufficient to supply only 20-30% of the total volume of cheese production (Jacob et al., 2011). Furthermore, there are groups of consumers who, for ethical or religious reasons (e.g. vegetarians, Judaism, and Islam), do not allow the use of animal rennet in cheeses. Based on these, dairy companies have highlighted the need to find alternative milk-clotting enzymes, that can satisfactorily replace animal enzymes in cheesemaking through the development of new technologies (Shabani et al., 2018). Suitable alternatives are recombinant bovine chymosin (banned in France, Germany, and the Netherlands) and microbial aspartic proteases (Egito et al., 2007). Recently, enzymes extracted from plants have become subject of increasing interest in dairy technology, as they can be used to produce vegetarian, Kosher, and Halal cheeses (Nicosia et al., 2022a). Several plant enzymes are currently tested for their use as milk-clotting enzymes. According to Guiama et al, (2021) *Solanum aethiopicum* could be successfully used to produce cheese with acceptable nutritional quality. Moreover, the use of *Carica papaya* leaf extract could be a viable alternative to animal rennet for improving odor in the production of fresh goat cheese without having any negative impact on color, texture or taste (Yasser et al., 2021). The aqueous extract obtained from *Cynara cardunculus* is one of the most utilized vegetable coagulants to produce sheep cheeses in the

Mediterranean basin (Fernández-Salguero et al., 2002). A study was conducted by Khan and Masud (2013) on the use of *Withana coagulans* extract for the production of ricotta with buffalo milk; however, results of the sensory evaluation highlighted a different appearance and flavour. In recent years, attention has turned to kiwifruit (*Actinidia deliciosa*) extract as a replacement of animal rennet (Nicosia et al., 2022b). Kiwifruit is appreciated for its beneficial effects on human health and contains large amounts of vitamin C, phenolic compounds, including caffeic acid and other chlorogenic derivatives, syringic acid, ferulic acid, gallic acid, salicylic acid (Sun-Waterhouse et al., 2009) and flavonoids, which are characterized by their anticancer and antioxidant properties (He et al., 2019). Kiwifruit represents a good source of actinidin: a cysteine protease able to hydrolyze milk caseins in conditions that are fully compatible with those used in industrial cheesemaking (Lo Piero et al., 2011). In the study conducted by Fguiri et al. (2021), the kiwifruit enzyme showed the highest suitability in cheesemaking using camel milk compared with pineapple (*Ananas comosus*) and ginger (*Zingiber officinale*) extracts. However, many of these plant enzyme preparations have been found to be unsuitable because both flavor and texture of cheeses are influenced by the high proteolytic activity of plant proteases, which leads to the production of short peptides responsible for bitterness in dairy products (Lo Piero et al., 2011; Egito et al., 2007). It has recently been demonstrated that the use of functional starter/adjunct culture is fundamental in cheese clotted with vegetable-derived enzymes. In detail, the use of specific strains of lactic acid bacteria with specific aminopeptidase activities is able to reduce the bitter taste produced by the high proteolytic activity of plant coagulants (Nicosia et al., 2023a; Nicosia et al., 2023b). This aspect is very important, because the acceptability of cheese to the final consumer arises from specific sensory characteristics, including aroma

and flavor. Sensory evaluation of cheese is a key step in assessing the ability of a vegetable rennet to successfully replace calf rennet (Amira et al., 2017). Some plant enzymes produce cheeses with similar sensory and organoleptic qualities to those made with calf rennet, while others contribute to a very different textures and flavors. The sensory evaluation of cheeses is the first driver behind purchase intention, more important than price, convenience, and health. Therefore, it is important to develop a product that provides desirable sensory characteristics (Liotta et al., 2019; Chiofalo et al., 2020; Short et al., 2021). Conventionally, human sensory evaluation is used to analyze the odor, taste, and color characteristics of cheese; however, this can have drawbacks, such as time-consuming, human objectivity, and thus non-repetitive analysis requiring professional tasters (Pearce and Gardner, 1998; Sinija and Mishra, 2009). In this regard, electronic eye, electronic nose, and electronic tongue can be considered as promising tools to evaluate and differentiate cheeses as they allow for objective and measurable data. E-Eye, E-Nose, and E-Tongue are three analytical systems that have been used as methods of food and feed quality assessment over the years (Kiani et al., 2016; Di Rosa & Leone, 2018a; Oteri et al., 2021). However, these instruments must be used properly and under optimal working conditions, because improper use could lead to limiting the amount of useful information obtainable from samples (Haddi et al., 2014). Several authors have applied them for the identification of changes in color, odor, and taste and in the quality of different types of food and beverages such as raw milk (Winqvist et al., 2005, Ciosek and Wroblewski, 2008; Di Rosa et al., 2018b; Lo Presti et al., 2023), fish (Huang et al., 2011; Trabelsi et al., 2021; Oteri et al., 2022), honey (Shafiee et al., 2014; Di Rosa et al., 2018c; Di Rosa et al., 2018d; Di Rosa et al., 2019; Russo et al., 2023), meat and meat products (Larrain et al., 2008, Valous et al., 2009).

Currently, there are limited data on the sensory characteristics of cheeses made with vegetable extract. The aim of this study was to evaluate the organoleptic profile of Sicilian cheeses obtained using kiwi extract, microbial coagulant and conventional animal rennet, as coagulant, through an artificial senses platform consisting of E-Eye, E-Nose and E-Tongue. In addition, microbiological analysis was carried out. Data from multiple sensors were combined with related information from associated databases.

5.3 Materials and methods

5.3.1 Vegetable coagulant preparation

The kiwifruit enzymatic extract, used as vegetable coagulant, was obtained from *Actinidia deliciosa* cv. Hayward fruits purchased from a local market in Catania (Sicily, Italy). In detail, kiwi fruits were pressed using a manual press and, after centrifugation at 8500 rpm for 20 minutes, the obtained aqueous extract was subjected to salting-out technique as described by Nicosia and co-workers (2023b). The precipitate, resulting after centrifugation at 9000 rpm for 20 minutes at 4 °C, was used as vegetable rennet in cheesemaking experiments.

5.3.2 Cheesemaking experiments

Three cheesemaking experiments (named C, H, and I) were carried out, in parallel, at the La Cava dairy farm (La Cava Srl, Randazzo, Italy). In detail, 90 L of whole cow's and sheep's milk, mixed in a 1:1 ratio, were heat treated at 72°C for 20 seconds, then cooled down to 38°C and split into three separate batches (30 L in each). After the inoculum of a commercial starter culture, kindly provided by Sacco System srl (Cadorago, Italy), the milk was clotted with calf rennet (0.02g/L, Caglificio Clerici Spa, Cadorago, Italy),

sample C; microbial coagulant (0.02g/L, Caglifificio Clerici Spa, Cadorago, Italy), sample I; or kiwifruit enzymatic extract (0.7g/L) inoculated with the *Lactococcus lactis* subsp. *lactis* bv. *diacetylactis* Q5C6 (1%) strain as debittering adjunct culture, sample H. The curd, obtained from each batch, was cut with a knife, sliced into small granules, measuring 5-6 mm in size, placed into cheese molds then pressed to pour out the whey. After immersion in a brine solution (saturated solution at 20°Bé at 12°C) for 15 min, the obtained cheese samples were stored under controlled conditions ($14 \pm 1^\circ\text{C}$, relative humidity of 90%) for 15 days.

5.3.3 Microbiological analysis

15 days old cheese samples were subjected to culture-dependent analysis following the method and using the culture media and condition reported by Pino et al. (2018). In addition, the protocol proposed by Carpino et al. (2017) was applied to investigate the presence of *Salmonella* spp. All media were purchased from Liofilchem (Roseto degli Abruzzi, Italy). The analysis was conducted in triplicate and results were expressed as \log_{10} CFU/g and standard deviation.

5.3.4 Chemical analysis

Samples were taken to the laboratory of the Department of Veterinary Sciences at the University of Messina and were analyzed. The chemical analyses of the cheese samples were conducted after 15 days of storage at controlled temperature of $14 \pm 1^\circ\text{C}$. For moisture content the gravimetric method was employed, where 5 grams of the samples were dried at $105 \pm 2^\circ\text{C}$ until constant mass was achieved (ISO 5534:2004). The Gerber-van Gulik method was utilized to

measure the total lipids content of the samples (ISO 3432:2008). The protein content was determined by the Kjeldahl method, which involved calculating the total nitrogen content of the samples. A conversion factor of 6.38 was used in the calculation, following the ISO 8968-1:2014 standard. For pH measurement, 10 grams of the sample were homogenized with 40 mL of distilled water. After 5 minutes, the mixture was filtered through cotton wool, and the pH was measured at a temperature of 25 ± 1 °C using a pH meter (MettlerDL25, Mettler-Toledo International Inc., Columbus, OH, USA). The total polyphenol content was assessed through spectrophotometry using Folin–Ciocalteu’s reagent, following the method outlined by Shetty et al. (1995). Approximately 50 mg of homogenized cheese was weighed, and 2.5mL of 95% ethanol was added, maintaining it at 0°C for 48 hours. Subsequently, 1mL of the resulting supernatant was transferred to a tube, combined with ethanol (95%) and ultrapure water, followed by the addition of Folin–Ciocalteu’s reagent (50%) and Na₂CO₃ (5%). Absorbances were measured at 760nm using a UV–visible spectrophotometer (UV-2401PC Shimadzu), with 95% ethanol serving as the analytical blank. A calibration curve was generated using appropriate dilutions of a gallic acid standard solution (95% in ethanol). Sterol profiles were determined using a Perkin Elmer Clarus 480 gas chromatograph, featuring a fused-silica capillary column (Zebron ZB-5MSi, L = 30 m, ID = 0.25 mm, FT = 0.25 μm) from Phenomenex (Torrance, CA, USA) and a Flame Ionization Detector (FID). Helium served as the carrier gas, circulating at a constant flow rate of 1 mL/min. The injector, operating in split mode with a split ratio of 1:10, and the detector were set at temperatures of 270°C and 300°C, respectively. Compound identification was achieved by comparing the gas chromatography retention times with those of pure standards analyzed under identical conditions. For the determination of fatty acids of cheese, a gas

chromatograph with an FID detector (Agilent Technologies 6890 N, Palo Alto, CA, USA) was employed, featuring a SP-2560 fused silica capillary column (100 m × 0.25 mm i.d. × 0.2 µm film thickness, Supelco, Inc., Bellefonte, PA, USA). Helium served as the carrier gas at a flow rate of 1 mL/min. The injection volume was 1 µl with a split ratio of 1:50. The column temperature was programmed with an initial isotherm at 70 °C for 2 minutes, followed by an increment of 15 °C/min to 155 °C (maintained for 25 minutes), and then an increment of 3 °C/min leading to a final isotherm at 215 °C for 8 minutes. This temperature program aligns with the protocol outlined by Tudisco et al. (2015). All analyses were performed in triplicate, and the results are reported as the mean value along with the standard deviation. All reagents and chemicals used were of analytical grade and obtained from Merck (Darmstadt, Germany).

5.3.5 Artificial senses analysis

Cheese samples were subjected to instrumental sensory analysis and evaluated for their color, odor and taste. For cheese color, a computer vision system (CVS), Iris Visual Analyzer 400 (Alpha M.O.S., Toulouse, France) was used. Image acquisition was performed as described in Di Trana et al. (2023) The Threshold value selected were as follows: R=73-186, G=0-162 and B=0-138.

An Electronic Nose (FOX 4000, Alpha M.O.S., Toulouse, France) equipped with 18 metal oxide sensors (P, T and LY type) was used to evaluate odor profile. For each sample, 10 replicates were prepared. In each vial, having the volume of 10 ml, 2 g of cheese was placed and analyzed using the following parameters: acquisition duration 120 s, acquisition time 1080 s, injection volume 500 µl, injection speed 500 µl/s, incubation time 1200 s, incubation temperature 60°C, flushing time 120 s, syringe temperature 70°C, fill

speed 500 $\mu\text{l/s}$; agitation speed 500 rpm.

An Electronic Tongue (α Astree, Alpha MOS, Toulouse, France) equipped with seven potentiometric sensors (ANS, PKS, CTS, NMS, CPS, ANS, SCS) was used to evaluate the taste profile. The E-Tongue was initially conditioned and calibrated with standard solution (HCl and NaCl), then with a solution as similar as possible to the one to be analyzed. The cheese samples were finely minced with a knife and divided into 4 g aliquots and analyzed according to Todaro et al. (2024). A beaker was prepared for each sample, and 20 consecutive measurements were taken for each. The sensors were rinsed with double-distilled water after each measurement. The last 10 seconds of signal acquisition related to the last 10 measurements were considered.

5.3.6 Statistical Analysis

The results of the sensor responses obtained from the individual instruments, E-Eye, E-Nose and E-Tongue, were analyzed by exploratory PCA performed by Alpha Soft V12.4 (Alpha-MOS, Toulouse, France). One-way ANOVA followed by Tukey post-hoc test ($p < 0.05$) was applied to microbiological and chemical data using the Minitab 16.0 software (Minitab Inc., State College, PA, USA).

5.4 Results

5.4.1 Microbiological analysis

The mean values and standard deviations of the main microbial groups detected in C (clotted with calf rennet), H (clotted using a kiwifruit enzymatic extract), and I (obtained using a microbial coagulant) cheese samples are shown in Table 1. Overall, *Escherichia coli*, *Listeria*, and *Salmonella* were never detected in the analyzed samples. Mesophilic bacteria, lactobacilli, and lactococci showed

similar viable count in both C and H samples. Differently, higher value of mesophilic bacteria and lower count of both lactobacilli and lactococci were detected in I samples. The yeasts count was similar among samples whereas statistically significant differences were observed for *Enterobacteriaceae* and staphylococci count (Table 1).

5.5 Chemical analysis

The mean values and standard deviations of pH, moisture, total lipids, protein, polyphenols, and sterols detected in C, H, and I samples are shown in Table 2 whereas the fatty acid profile is displayed in Table 3. Concerning chemical data, significant differences were observed among samples. In particular, compared to C and I samples, cheeses clotted with kiwifruit enzymatic extract (H samples), showed lower values of pH, total lipids, protein, whereas highest moisture and polyphenols were detected. Stigmasterol, campesterol, and β -sitosterol were detected only in H samples which also showed the lowest content of cholesterol. Concerning fatty acids composition, no statistically significant differences were observed for monounsaturated fatty acid (MUFA) as well as omega 3 fatty acid (n3) and omega 6 fatty acid (n6) series. Cheeses clotted using the kiwifruit enzymatic extract (H samples) showed the highest content of saturated fatty acids (SFA) and the low percentage of polyunsaturated fatty acids (PUFA).

Table 1. Microbial counts, reported as log₁₀ cfu/g and standard deviation, of the main microbial groups detected on experimental cheese samples.

	C	H	I
Mesophilic bacteria	6,06 ± 0.40 ^b	6,05 ± 0.22 ^b	6,85 ± 0.12 ^a
Lactobacilli	6,87 ± 0.10 ^a	6,90 ± 0.21 ^a	6,14 ± 0.04 ^b
Lactococci	6,75 ± 0.40 ^a	6,78 ± 0.25 ^a	6,00 ± 0.10 ^b
Yeast	3,65 ± 0,20 ^a	3,71 ± 0,41 ^a	3,73 ± 0,12 ^a
Enterobacteriaceae	2,20 ± 0,04 ^b	1,55 ± 0,10 ^c	3,15 ± 0,11 ^a
Staphylococci	2,22 ± 0,05 ^b	1,51 ± 0,15 ^c	3,34 ± 0,24 ^a
<i>E. coli</i>	<1	<1	<1
<i>Salmonella</i> spp.	<1	<1	<1
<i>Listeria</i> spp.	<1	<1	<1

Data are reported as average values and standard deviations of three replicates. ^{a-b} Different lowercase letters indicate

statistically significant differences ($p < 0.05$) among samples. C, cheese clotted with animal rennet; H, cheese clotted with kiwifruit enzymatic extract; I, cheese clotted with microbial coagulant

Table 2. Chemical profile of experimental cheese samples obtained using different milk-clotting enzymes.

	C	H	I
pH	6.10 ± 0.01^b	5.88 ± 0.05^c	6.40 ± 0.04^a
Moisture (g/100g)	39.91 ± 0.08^b	47.84 ± 0.28^a	40.52 ± 0.13^b
Total lipids (g/100g)	32.21 ± 0.44^a	28.90 ± 0.44^b	31.90 ± 0.22^a
Proteins (g/100g)	24.90 ± 0.31^a	20.31 ± 0.59^b	25.55 ± 0.67^a
Polyphenols (mg/kg)	153.88 ± 0.02^b	422.90 ± 0.05^a	148.70 ± 0.15^b
Sterols			
(mg/100g of cheese)			
Cholesterol	93.32 ± 0.60^a	55.41 ± 0.18^b	90.23 ± 0.14^a
Stigmasterol	nd	0.19 ± 0.05	nd
Campesterol	nd	0.16 ± 0.07	nd

β -sitosterol

nd

0.55±0.15

nd

Data are reported as average values and standard deviations of three replicates. Different letters indicate statistical significant differences ($p < 0.05$) among samples. C, cheese clotted with animal rennet; H, cheese clotted with kiwifruit coagulant; I, cheese clotted with microbial coagulant. nd, not detected

Table 3. Fatty acid composition of experimental cheese samples

	C	H	I
SFA	69.20 ± 0.06 ^b	69.55 ± 0.10 ^b	71.22 ± 0.11 ^a
MUFA	23.39 ± 0.09 ^a	23.43 ± 0.07 ^a	23.40 ± 0.16 ^a
PUFA	4.00 ± 0.02 ^a	3.94 ± 0.05 ^a	3.11 ± 0.03 ^b
n3	1.24 ± 0.10 ^a	1.25 ± 0.03 ^a	1.21 ± 0.03 ^a
n6	2.41 ± 0.02 ^a	2.48 ± 0.04 ^a	2.38 ± 0.03 ^a
n6/n3	1.64 ± 0.14 ^a	1.65 ± 0.02 ^a	1.62 ± 0.02 ^a

Data are reported as percentage. SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty

acid; n3, omega 3 fatty acid series; n6, omega 6 fatty acid series. Data are reported as average values and standard deviations of three replicates. Different letters indicate statistical significant differences ($p < 0.05$) among samples. C, cheese clotted with animal rennet; H, cheese clotted with kiwifruit coagulant; I, cheese clotted with microbial coagulant.

5.6 *Artificial senses analysis*

Results from each electronic instrument are shown in figures 1, 2 and 3. In the PCA plot of the E-Eye (Fig.1) the first two components (PC1 and PC2) account for 97 percent of the total variance between the measurement of the samples. The PCA plot shows an overlap of groups C and H to the left of the graph with a great number of color codes, from the darkest to the lightest, while group I separates sharply by positioning itself to the right of the graph highlighted by the color codes 2181-2437-2438-2454. In the PCA plot of the E-Nose (Fig. 2) the first two components (PC1 and PC2) account for 100 percent of the total variance between the measurement of the samples. As for E-Eye, PCA also shows an overlap of group C and H, placing to the right of the graph. The volatile profile of group H does not differ from group C, however group H shows less internal variability. The I group diverges from the C and H groups, along the PC1 axis, placing it to the left of the graph. For the taste profile, in the PCA plot of the E-Tongue (Fig. 3) the first two components (PC1 and PC2) account for 100 percent of the total variance among the measurement of the samples. The PCA plot shows a taste separation of the three groups; specifically, group I is placed on the left side of the graph, while group H on the right top and group C on the right and bottom of the graph. Although E-Tongue discriminates the three cheese groups, all the sensors are biased toward group I.

To increase the probability of correct classification, a mid-level data fusion was implemented and the single matrix with data from all sensors was subjected to PCA. In addition, data of nutritional characteristics, fatty acid classes, total content polyphenols, tocopherols and phytosterols were included. In the PCA graph (Fig. 4) the first two components (PC1 and PC2), represent the 100 percent

of the total variance between the measurement of the samples. Observing the PCA (Fig. 4), the I group is at the bottom left of the graph, the H group is at the top of the graph, and the C group is at the bottom right of the graph. Observing the direction of the sensors of the sensory instrumentation, we can see that group I is characterized by sensors T40/1 (E-Nose), ANS and CTS (E-Tongue), 2439 (E-Eye), group H is characterized by sensors 2182 and 2166 (E-Eye), while sensors LY2/gCT and LY2/AA (E-Nose), 2454 (E-Eye) are heading toward group C. As for chemical analysis, SFAs and MUFAs head toward group I. Toward group H, the polyphenols, Campesterol, Stigmasterol and β -sitosterol head. While the n3, n6, n6/n3, and PUFAs are between the H and C groups. The α -tocopherol, γ -tocopherol, δ -tocopherol, and cholesterol do not direct to a specific group.

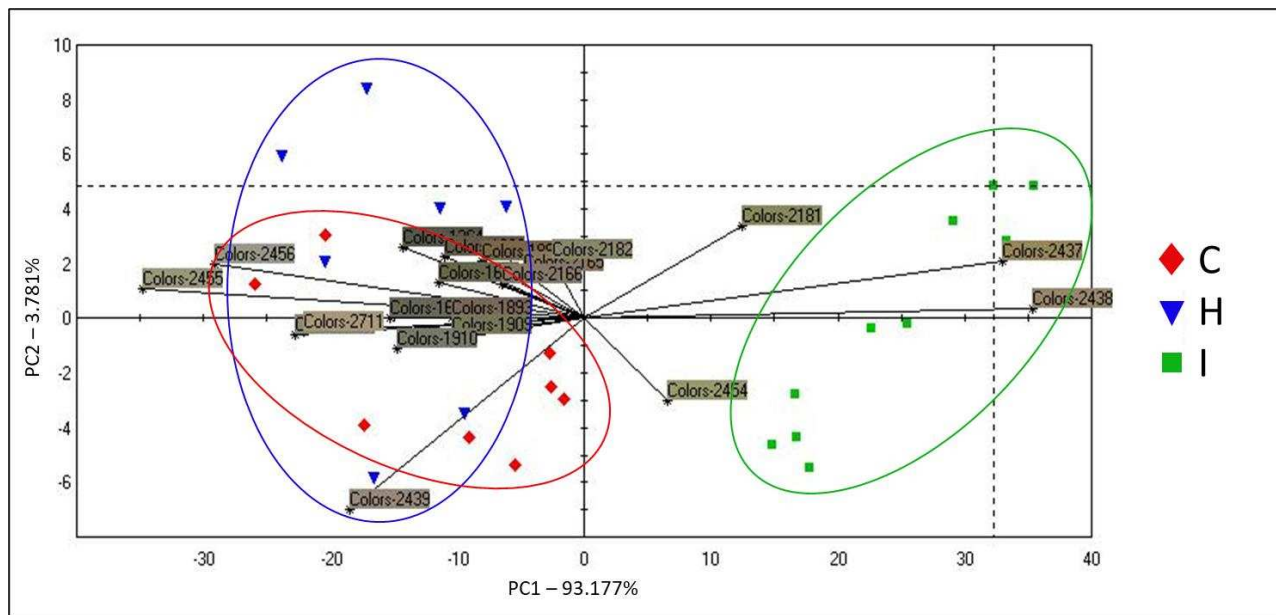


Fig. 1 Principal Component Analysis plot for the E-Eye (C, red; H, blue; I, green) and loading vectors.

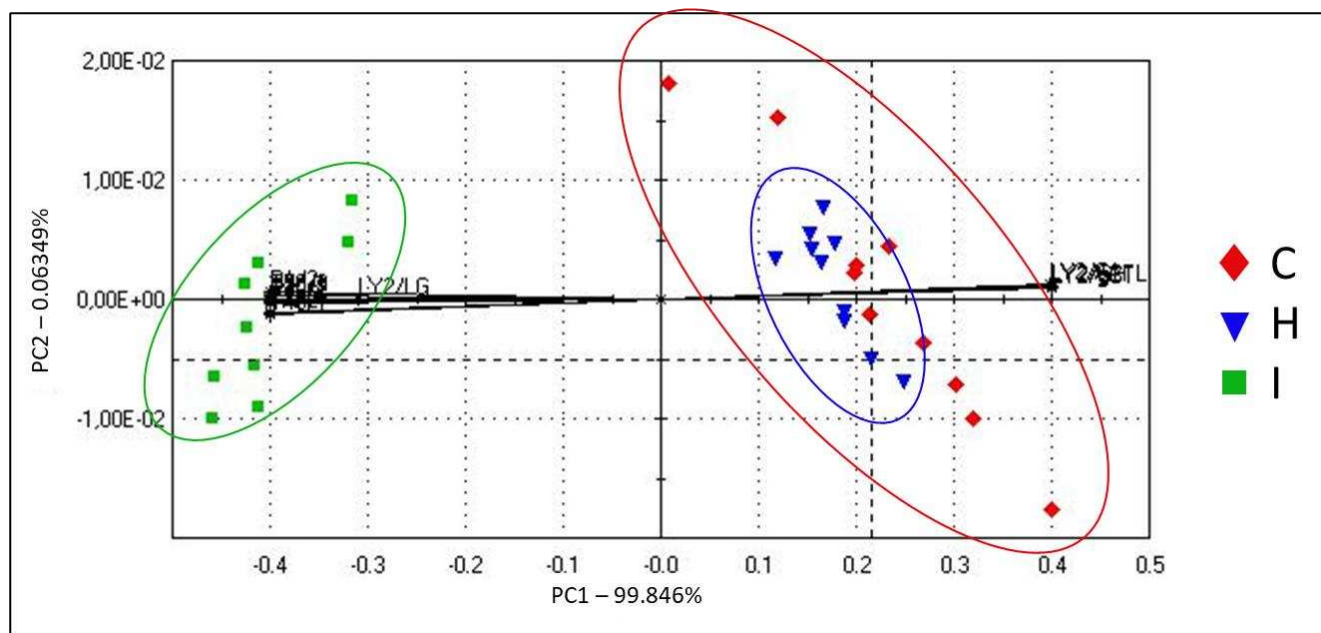


Fig. 2 Principal Component Analysis plot for the E-Nose (C, red; H, blue; I, green) and loading vectors.

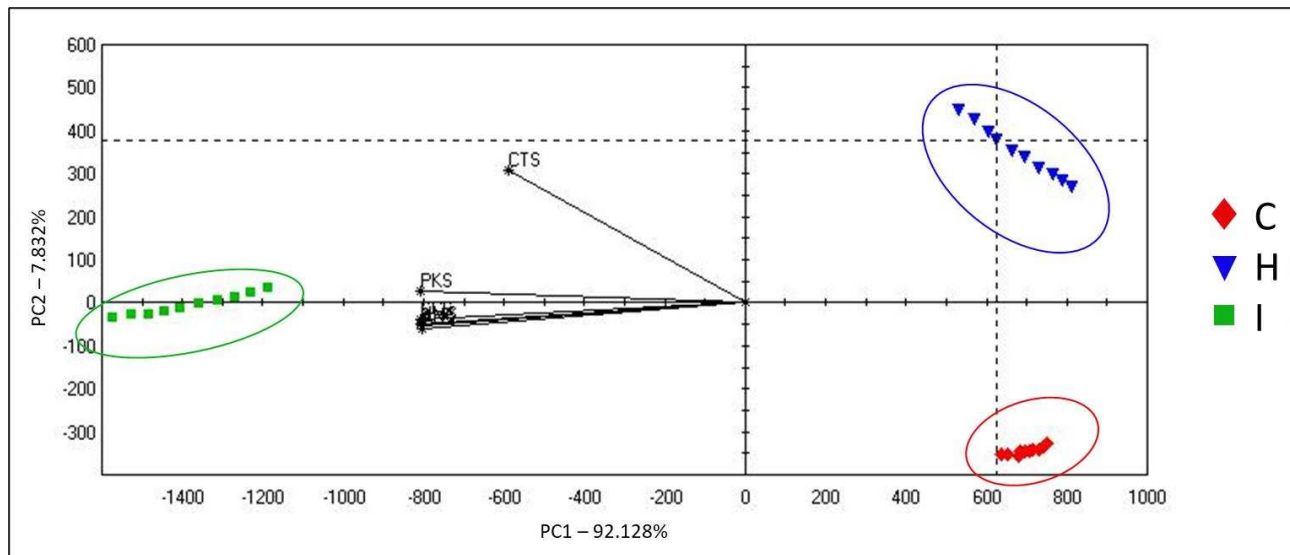


Fig. 3 Principal Component Analysis plot for the E-Tongue (C, red; H, blue; I, green) and loading vectors.

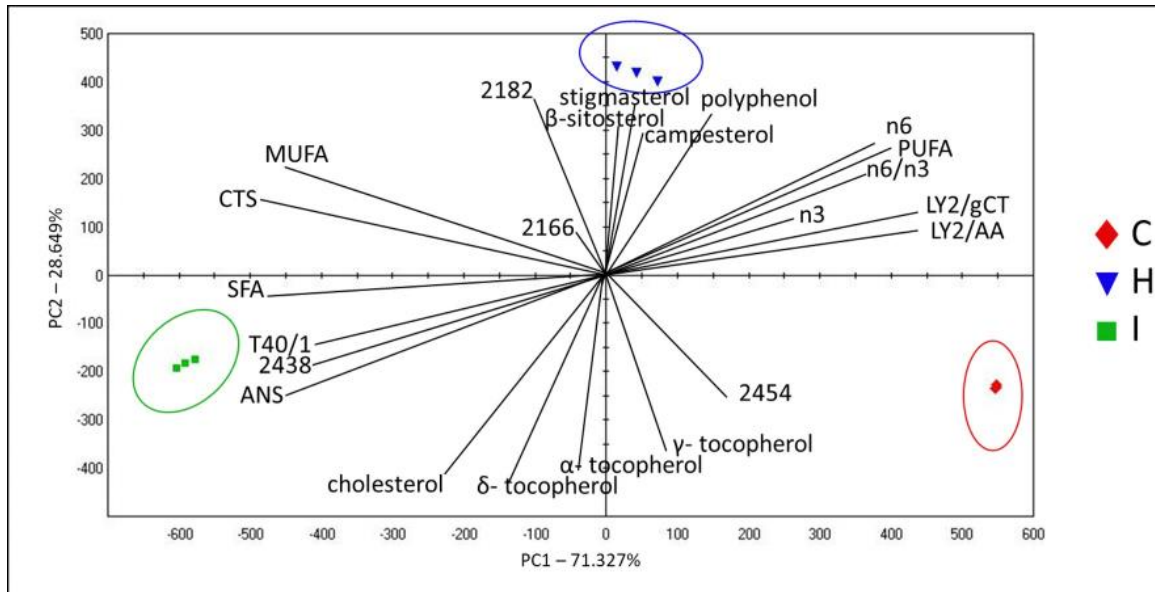


Fig. 4 Principal Component Analysis plot for the Medium fusion (C, red; H, blue; I, green) and loading vectors of selected variables.

5.7 Discussion

In the present study, an artificial senses platform consisting of E-Eye, E-Nose and E-Tongue was applied to evaluate sensorial characteristics of Sicilian cheese, made using a plant-derived milk-clotting enzyme, compared to those of cheese produced by calf and microbial coagulant. In addition, microbiological and chemical assessment were carried out. An interesting aspect is represented by the polyphenols content; sample coagulated with kiwifruit rennet showed a higher content of polyphenols than animal and microbial coagulant cheese. This result agrees with Serra et al. (2020) who investigated the functional properties, related to polyphenols, of sheep and buffalo milk cheese coagulated with kiwifruit pulp. It is noteworthy that polyphenols, which are present in kiwifruit, have been widely recognized for their positive impact on human health. They possess anti-cancer, anti-diabetic, anti-aging, anti-hypertensive, anti-asthmatic, and cardio-protective properties (Pandev et al., 2009). Additionally, polyphenols offer protection against the oxidation of LDL cholesterol and other lipids in the bloodstream (Heinonen et al., 1998). These benefits also support the utilization of kiwifruit in cheese manufacturing to improve the overall nutritional profile and functional characteristics of the product. From the microbiological point of view the absence of *E. coli*, *Listeria* and *Salmonella* reflects the hygienic conditions of the starting milk and demonstrates its applicability for cheesemaking. However, it is interesting to note that in samples C and H the count of lactobacilli and lactococci was higher than in sample I; this probably had a positive effect against pathogens. In fact, in the process of cheese fermentation, the majority of lactic acid bacteria (LABs) exhibit antibacterial properties by generating lactic acid and

lowering the pH in the substrate. The acidic environment represents a hurdle to the growth of many bacteria, resulting in a lower value of mesophilic bacteria, *Enterobacteriaceae*, staphylococci in samples C and H compared to sample I. Similar results were reported in a previous work of Nicosia et al. (2023b), in which reduced microbial counts for mesophilic bacteria and staphylococci were observed in vegetable-clotted cheeses with higher concentration of lactococci and therefore lower pH. Regarding sensorial parameters evaluated through E-Eye, E-Nose and E-Tongue equipment, data from E-Nose revealed how P- and T-type sensors go to characterize group I. P-type sensors are sensitive to ketones and T-type to alcohol compounds (Upadhyay et al., 2017). Ketones are formed by the enzymatic oxidation of fatty acids to β -keto acids and then their decarboxylation to methyl-ketones are compounds present at high concentrations in cheeses made with pasteurized milk and a specific starter culture (Castillo et al., 2007). Furthermore, the position of the T-type sensors could be explained by the formation of secondary alcohols, formed by enzymatic reduction of the respective methyl ketones (Delgado et al., 2011). The cheeses made with animal and vegetal coagulants (C and H group), were identified by the LY group sensors which, instead, respond to carboxylic acids, important components in the aroma of cheeses (Demirci et al, 2021), and to aldehydes, deriving from the autoxidation of carboxylic acids with a role in the flavor development of cheese (Dunn HC & Lindsay R, 1985; Muñoz N. et al., 2003). Moreover, the LY sensors respond to alcohols, probably the branched-chain primary alcohols formed by reduction of aldehydes derived from leucine (Delgado et al., 2011). In general, the substances that most affected the aroma of the cheeses were the same in the C and H group and could be related to the high presence of lactobacilli and lactococci. It is well known that the metabolism of free amino acids (FAAs) by LABs, produces aldehydes, alcohols, carboxylic acids, amines, and sulfur

compounds (Kilcawley et al., 2017). Aromatic amino acids, branched-chain amino acids, methionine, and aspartic acid undergo a transamination reaction catalyzed by amino acid aminotransferase, converting them into α -keto acids. Subsequently, these α -keto acids are further metabolized into branched-chain and aromatic aldehydes, acyl-CoA, hydroxy acids, and methanethiol. The resulting organic acids can undergo esterification with alcohols, contributing to an enriched aroma of the cheeses (Delavenne et al., 2012; Belguesmia et al., 2014). Furthermore, cheese color is an attribute that is often overlooked but can become important when pleasant flavor notes are missing. In our study the use of kiwifruit rennet did not affect the final color of the cheese, according to Garcia et al., (2012) that, using a CIE Lab system, also found no difference in color between cheeses produced with three types of coagulant (microbial, vegetable and animal). However, group I showed the presence of distinctive color codes that are not present in groups C and H. Groups C and H appear to have the same colors even though group H has the darkest colors. These results, which could be due to the greater presence of polyphenols found in cheeses made with kiwifruit coagulant (Han et al., 2011), are however not significant, contrary to what was found by Saraiva et al. (2019) in cheeses in which the use of Yerba mate have attributed light green shades. An important contribution to cheese flavor is made by smaller peptides and free amino acids, as well as compounds derived from the catabolism of free fatty acids. It is well known how the use of vegetable rennet contributes negative notes in taste, due to the higher proteolytic activity of vegetable coagulants compared to chymosin (Galan et al., 2012). The high proteolytic activity, leads to the production of short peptides, generating bitter and sour taste. The use of microbial coagulant also brings bitter taste in cheeses. In previous work (Ahmed et al., 2016; Vishwanatha et al., 2010), *B. stearothermophiluse* and fungal curds have been shown to

cause bitter taste defects in several cheeses. In a study by Zhao et al. (2019), microbial coagulant isolated from glutinous rice wine was used for Cheddar cheese production and compared with a commercial rennet. From the results of the sensory evaluation, the cheese with glutinous rice wine rennet showed no significant differences in terms of preference, compared to the cheese made with commercial rennet. Both cheeses showed good acceptability. These results are not in agreement with those obtained by E-Tongue. In fact, it was the only instrument that discriminates among the three groups of cheeses, highlighting different flavor notes induced by the different rennet used.

For the E-Tongue, the AHS, CTS, NMS, ANS and SCS sensors specifically respond to sour, salty, umami, sweet and bitter tastes, respectively, while PKS and CPS function as universal (Zhu et al., 2022). In particular, looking at the direction of the sensors, they point toward group I cheeses. We could hypothesize that group I is characterized by a set of tastes that distinguish cheeses made with microbial coagulant, according to Zhao et al. (2019), who found that the high level of FAA umami in cheese produced with microbial coagulant isolated from glutinous rice wine increase the intensity of other flavors, improving the taste of the cheese (Jinap & Hajeb, 2010). Results obtained from chemical and sensorial data fusion showed that cheeses made with kiwifruit coagulant are more similar to cheeses made with animal rennet than those made with microbial coagulant. However, group H differs from group C in the presence of the phytosterols Campesterol, Stigmasterol and β -sitosterol, which are the most abundant sterols in kiwifruit (Piironen et al., 2003). Sterols are produced by plants and have functions like those of cholesterol in humans. Dietary supplementation of plant sterols plays an important role in human metabolism, as they can attenuate the intestinal absorption of cholesterol (Gylling et al., 2014). In addition,

polyphenols and their metabolites can act as activators or inhibitors of bacterial growth, and this depends on their chemical structure (Reguant et al., 2000). These metabolites exhibit a selective ability to inhibit the growth of pathogens while promoting the growth of commensal bacteria, consequently, influencing the composition of the cheese microbiota (Cueva et al., 2010; Laparra and Sanz, 2010). In the present study, the high polyphenols and phytosterols content of the H sample seems to explain the lower level of stafilococci and Enterobacteriaceae respect to samples C and I, allowing an high dominance of lactobacilli and lactococci. Thus, the use of kiwifruit extract is beneficial because it helps improve the functional characteristics of cheese (Serra et al., 2020). In light of the results obtained from medium fusion, after skimming the irrelevant data, it was observed in more detail that the H group is characterized by light colors; in fact, color codes 2182 and 2166 correspond to a shade of yellow and a shade of blue-turquoise, respectively. Probably the color was influenced by the presence of polyphenols. Conversely, groups I and C were characterized by the color codes 2139 corresponding to a cyan-blue shade and 2454 corresponding to a medium-dark shade of turquoise, respectively. Therefore, groups I and C are characterized by darker color shades, but still of the same hue. Another interesting result is the sensory response of E-Nose. Sensor T40/1 characterizes group I; moreover, this group is characterized by a higher presence of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA). In contrast, sensors representing short-chain fatty acids (LY) and polyunsaturated fatty acids (PUFA) are directed toward groups C and H. The presence of short-chain fatty acids in cheese contributes to its sensory qualities due to their lower threshold of perception compared to long-chain fatty acids (Laskaridis et al., 2013). Group I was produced with microbial-derived coagulant and has had its own market for years. Nevertheless, it is the one with high level of saturated

fatty acids (SFAs). SFAs, which are most abundant in products of animal origin, such as milk and dairy products, are considered one of the major risk factors for human disease (Ferrucci et al., 2006). In the study conducted by Torres et al. (2022), Paipa cheese, obtained from the recombinant, microbial-derived enzyme chymosin EC 3.4.23.4, had a high content of saturated fatty acids and n3, n6 and n9. Indeed, the relevance of MUFAs should be emphasized, as from a nutritional point of view, consumption of these cheeses provides the essential benefits to protect human health (Renes et al., 2019). Monounsaturated fatty acids are important because they are useful as a source of energy, reduce blood concentrations of low-density lipoprotein, and prevent cardiovascular disease (De Souza et al., 2015).

5.8 Conclusions

In conclusion, this work shows how the use of kiwifruit extract for cheesemaking allows to obtain a final product with an organoleptic profile comparable to those coagulated with traditional animal rennet. However, the cheese coagulated with coagulant derived from kiwifruit showed higher content of phytosterols and polyphenols compared to cheeses made with microbial and animal coagulant and lower presence of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA), allowing a healthy and safe cheese.

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6 Other activities: Formulation of germinated brown rice fermented products functionalized by probiotics

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6.1 Abstract

Germinated brown rice is considered a functional food in relation to the presence of beneficial nutrients and bioactive compounds in considerable amounts. The present study, evaluated, for the first time, the suitability of germinated brown rice for the production of value-added fermented products functionalized by the addition of probiotics. Four different fermented products with (EYGG, EYBB536, EYBB12, and EYM) and without (EY) probiotics addition were formulated. Microbiological and chemical profiles, presence of bioactive compounds, antioxidant activity, sensory attributes, and shelf-life during refrigerated storage were evaluated. Results showed that the fermentation process determined the improvement of both bioactive compound profile and anti-inflammatory properties of germinated brown rice. All the fermented products were microbiologically stable during refrigerated storage at +4 °C for 28 days. In addition, a high count of both lactobacilli and bifidobacteria was achieved during the shelf-life, indicating the suitability of the germinated brown rice as a probiotic carrier. Based on the sensory profile, high acceptability scores were attributed by panelists to the germinated brown rice experimental products. Based on the aforementioned results, germinated brown rice can be processed as a new fermented formulation with potential health benefits.

6.2 *Introduction*

The increasing demand for high-quality foods by health-conscious consumers has managed both the scientific community and the food industry to develop new products with enhanced nutritional and functional properties along with appreciable sensory quality (Guiné, Florença, Barroca, & Anjos, 2020). In addition, due to both lactose intolerance and allergies to milk proteins as well as to the spread of people who follow vegetarian and vegan diets, an increase in the demand for vegetable-based products was registered. In this context, cereals are considered a promising alternative to milk and milk-based foods. Among these, increasing attention was paid to brown rice which is characterized by a balanced and complete nutritional profile due to the presence of essential fatty acids, proteins, fibres, vitamins, antioxidants, and key phytochemicals (Beaulieu, Reed, Obando-Ulloa, Boue & Cole, 2020b; Gong et al., 2017). In particular, tocopherols, tocotrienols, oryzanol, B vitamins, phytosterols (β -sitosterol, campesterol, and stigmasterol), carotenoids, and beneficial phenolics confer health-promoting relevance to brown rice. The aforementioned compounds boast antioxidant properties, the ability to scavenge free radicals, aptitude to improve the immune system and reduce the risk of both heart disease and cancer development (Okarter, Liu, Sorrells & Liu, 2010; Liu, 2007; Xu, Hua & Godber, 2001; Qureshi, Mo, Packer & Peterson, 2000).

The germination treatment is one of the main technologies applied to brown rice to improve textural and organoleptic qualities, flavor components, and phytochemical bioavailability (Cáceres, Martínez-Villaluenga, Amigo, & Frias, 2014). Several studies had shown that the germination process allows to an increase in the level

of specific bio-functional components, such as γ -aminobutyric acid (GABA), lysine, B-group vitamins, and several antioxidants (e.g. γ -oryzanol, vitamin E, and phenolic compounds) (Han, Arijaje, Jinn, Mauromoustakos, & Wang, 2016; Cho, & Lim, 2016; Shao & Bao, 2015; Zhang et al. 2014; Wu, Yang, Toure, Jin, & Xu, 2013; Kim et al., 2012). In particular, GABA exerts health-promoting properties, including the regulation of blood pressure; the stimulation of immune cells; the alleviation of pain, anxiety, and sleeplessness; the reduction of cancer cell proliferation; the prevention of diabetes, which were demonstrated in animal feeding studies (Esa, Abdul-Kadir, Amom, & Azlan, 2013; Imam, Azmi, Bhangar, Ismail, & Ismail, 2012; Jung, Kim, Hwang, & Ha, 2007; Ardiansyah et al., 2006). In addition, brown rice phenols, such as ferulic and caffeic acids, can exert chemopreventive action preventing the growth of human breast and colon cancer cells (Hudson, Dinh, Kokubun, Simmonds, & Gescher, 2000).

Along with the enhancement of health-promoting properties, the germination process makes brown rice suitable for the production of different food products (e.g. gluten-free noodles, beverages, cookies, and bread) (Wang et al., 2020; Cáceres, Peñas, Martínez-Villaluenga, García-Mora, & Frías, 2019; Zhang, Liu, Wang, Liu, & Lan, 2019; Wu, Yang, Chen, Jin, & Xu, 2011). In particular, brown rice beverages represent one of the main commercially available and their consumption is increasing worldwide (Paul, Kumar, Kumar, & Sharma, 2020; Mäkinen, Wanhalinna, Zannini, & Arendt, 2016). Up to now, several studies were focused on the evaluation of different methods exploitable to make brown rice beverages as well as on their effects on physicochemical parameters (Beaulieu, Reed, Obando-Ulloa, & McClung, 2020a). Even though the fermentation process, like germination, can improve nutritional value, sensory and functional properties of cereals, such as germinated brown rice, scant

is the literature related to the formulation of fermented brown rice products (Montemurro, Pontonio, Coda, & Rizzello (2021); Cáceres, Peñas, Martínez-Villaluenga, García-Mora, & Frías 2019). According to that, the present study aimed to formulate fermented brown rice products with enhanced nutritional and health-promoting properties starting from two commercially available brown rice drinks. Both drinks were obtained through two different technological processes and were analysed from both physicochemical and microbiological points of view.

6.3 Material and methods

6.3.1 Germinated brown rice

Two different germinated brown rice drinks (GBR1 and GBR2), kindly provided by Mr. Bio Food srl (Crespadoro, Italy) were used in the present study. In detail, GBR1 was produced by sprouting brown rice seeds in water at 25 ± 2 °C for 48 hours. The sprouted brown rice grains were steamed at 121 °C for 15 minutes, to reduce the microbial load present in the composition, and then subjected to micronization in a colloidal mill, to obtain particles with an average size between 10 and 100 µm. The micronization process was carried out in presence of water to limit the overheating of nutrients and, therefore, their thermo-degradation and/or oxidation. The obtained composition was incubated for 1 hour at 37 °C in presence of 2 g/Kg of alpha- and beta-amylase enzymes to transform the starch into malts and simple sugars. GBR2 was obtained following the process previously described with the exception of steamed and incubation with enzymes, which were not applied.

6.3.2 Probiotic strains and starter cultures

Bifidobacterium longum BB536 (ATCC BAA-999), *Bifidobacterium bifidum* Bb-12 (kindly provided by Chr. Hansen Holding A/S (Denmark)), and *Lactocaseibacillus rhamnosus* GG (ATCC 52103) were used in the present study as probiotic strains. The commercially available starter cultures Cryofast SST 31 (*Streptococcus thermophilus*) and Lyofast SY 1 (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*), kindly provided by Sacco System srl (Cadorago, Italy), were used as deep-frozen and freeze-dried cultures, respectively. In addition, the YoFlex® YF-L02 DA, a thermophilic lactic acid culture, kindly provided by Chr. Hansen Holding A/S (Denmark), was used as frozen pellet.

6.3.3 Chemical characterization of germinated brown rice

GBR1 and GBR2 samples, obtained from three different germination batches (a, b, and c), were subjected to chemical characterization. In detail, moisture, protein, fat, fiber, and ash were determined following AOAC (2016) methods. Sugars were detected by high-performance liquid chromatography with pulsed amperometric detection (HPLC-PAD). Carbohydrates were calculated by difference. Energy was determined using standardized conversion factors (4.0 kcal/g for proteins and carbohydrates, and 9.0 kcal/g for fats) (FAO, 2002). pH was measured using a digital pH-meter (MettlerDL25, Mettler-Toledo International Inc.). Each analysis was performed in triplicate.

6.3.4 Detection of bioactive compounds

All reference standards were purchased from Sigma-Aldrich

unless otherwise indicated. They were stored at -20°C and kept in the dark until use. The samples were collected and stored at -20°C until mass analysis. Selected compounds were: Butyric acid, Phytic acid, Oryzanol, GABA, Inositol, Lactic acid. Samples were prepared according to the protocol reported by Cáceres and co-workers (2019, 2017). The active component was identified by Perkin Elmer – ABSciex ultra liquid chromatography-mass spectrometry (UPLC-Ms/Ms) system. The column used were an Phenomenex Luna $3\ \mu\text{m}\ \text{NH}_2\ 100\text{A}$, LC Column $100 \times 2\ \text{mm}$ and an Phenomenex Luna C18, $5\ \mu\text{m}$, $15 \times 0.1\ \text{cm}$. ESI-Ms/Ms analysis was performed in negative and positive mode to find the best operative conditions. Negative polarity was used in full scan mode and the mass range was set at 100-1500 Da. The condition of UPLC were as follows: from 0 to 2 min at 60:40 v/v; from 2 to 30 min acetonitrile was changed to 100% linearly; from 30 to 40 min the system was reconnected to the initial conditions. A total volume of $10\ \mu\text{l}$ was injected. The mass spectrometry settings are drying gas (N_2) flow rate (10 l/min); Curtain gas 30; IonSpray Voltage (IS) ± 4500 ; Ion Source Gas1 (GS1) 30.0; Ion Source Gas2 (GS2) 60.0 and Interface Heater ON; Declustering Potential (DP) ± 50.0 ; Focusing Potential (FP) ± 400.0 and Entrance Potential (EP) ± 10.0 .

6.3.5 Microbiological analysis of germinated brown rice

Germinated brown rice GBR1 and GBR2 samples, obtained from three different germination batches (a, b, and c), were subjected to microbiological analysis. In detail, total mesophilic bacteria, lactic acid bacteria, lactococci, yeasts, Enterobacteria, faecal coliforms, staphylococci, *Escherichia coli*, and *Listeria* spp. counts were performed according to the method and using the culture media and conditions reported by Randazzo and co-workers (2021). In addition,

Brilliance Salmonella agar (BSA), supplemented with Salmonella selective supplement, incubated at 37 °C for 24 ± 3h, was used for *Salmonella* spp. count according to ISO 6579:2002+A1:2007 standard. All media were purchased from Oxoid (Italy). Analyses were performed in triplicate and results were expressed as mean log₁₀ cfu/ml and standard deviation.

6.3.6 Isolation and identification of autochthonous lactic acid bacteria

In order to in-depth study the autochthonous lactic acid population of GBR2 samples, 20% of the total number of colonies recovered on MRS and M17 agar plates were randomly selected. Isolates were purified by streaking three times and then subjected to microscopic observation, catalase, and Gram reaction before storing at -20°C in liquid culture containing 20% of glycerol (v/v). Overall, 90 isolates were obtained and 80 of them were found gram-positive and catalase-negative. Isolates and reference strains, listed in Supplementary Table 1, were subjected to total DNA extraction following the protocol described by Pino and co-workers (2019) and Randazzo and co-workers (2015). DNA concentration was assessed by measuring optical density using Fluorometer Qubit (Invitrogen, Carlsbad, CA, USA). The LAB isolates were clustered by PCR-RFLP analysis according to Pino and co-workers (2018). One representative member of each PCR-RFLP cluster was subjected to 16S rRNA gene sequencing. The identification at the species level was done by comparing the obtained sequences with known 16S rRNA gene sequences in the NCBI (National Center for Biotechnology Information) (www.ncbi.nlm.nih.gov) database and those of the Ribosomal Database Project (<http://rdp.cme.msu.edu/index.jsp>) by using the BLAST algorithm. Sequences with a percentage identity of

98.7% were considered to belong to the same species.

6.3.7 Abilities of starter cultures to acidify and coagulate germinated brown rice

To test acidifying activity, a 50% (v:v) mixture of both GBR1 and GBR2 was inoculated with YoFlex or with SST 31 and SY 1 starter cultures in a 1:1, 1:2, or 2:1 ratio and incubated at 42 °C. The pH changes were monitored at regular intervals up to 14 h using a pH meter (H19017, Microprocessor, Hanna Instruments, Ronchi di Villafranca, Padovana, Italy). Coagulation was visually evaluated till 8 h of incubation.

6.3.8 Manufacture of experimental fermented products

Based on previous results, the experimental fermented products were formulated using a 50%(v/v) mixture of GBR1 and GBR2 samples inoculated with the starter cultures SST 31 and SY 1 in a 1:1 ratio. After fermentation at 42 °C for 12h, probiotic strains were inoculated at a final cell density of 9 log cfu/ml. Overall, five different fermentation batches were obtained namely: EYGG inoculated with *Lactocaseibacillus rhamnosus* GG (ATCC 52103); EYBB536 inoculated with *Bifidobacterium longum* BB536 (ATCC BAA-999); EYBB12 inoculated with *Bifidobacterium animalis* subsp. *lactis* BB-12; EYM inoculated with a mixture of the aforementioned probiotic strains; and EY un-inoculated with probiotics. Each experimental fermentation was performed in triplicate.

6.3.9 Microbiological stability of the experimental fermented products during storage

Microbial stability and *shelf-life* of the experimental fermented

products EY, EYGG, EYBB536, EYBB12, and EYM, stored at +4°C ($\pm 1^\circ\text{C}$), were weekly monitored for 28 days. Cell densities of yeasts and moulds, lactococci, Enterobacteria, faecal coliforms, staphilococci, *E. coli*, *Listeria* spp., and *Salmonella* spp., were determined as previously described (Randazzo et al., 2021). In addition, BSM Agar (Oxoid, Italy), incubated at 37 °C for 48h under anaerobic conditions, was used for bifidobacteria enumeration whereas lactobacilli were cultured on Rogosa Agar (Oxoid, Italy) incubated at 35 °C for 48-72h under aerobic conditions.

6.3.10 Chemical and bioactive compounds analysis of fermented products

The EY, EYGG, EYBB536, EYBB12, and EYM fermented products were subjected to moisture, protein, fat, fiber, ash, and carbohydrates determination as described before. In addition, the profile of the bioactive compounds (GABA, butirric acid, phytic acid, inositol, and oryzanol) was investigated as previously described.

6.3.11 Anti-inflammatory properties

Human colon immortalized cell lines (Caco-2) were used to investigate the anti-inflammatory properties of EY, EYGG, EYBB536, EYBB12, and EYM fermented products. The cell line was purchased from the American Type Culture Collection (Rockville, MD, USA). CaCo-2 cells were cultured in either 75 or 150 cm² flasks, in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (pen/strep), and 2 mM L-glutamine. Cells were cultured at 37°C and 5% CO₂. To

simulate the inflammation, the cells were further incubated with or without Lipopolysaccharides (LPS) at the concentration of 100ng/ml for 2 hours with the same medium (DMEM, 10% FBS) 34308769-34269367. Further, we treated the cells also with 10% of EY, EYGG, EYBB536, EYBB12, and EYM fermented products to test their effects. Cells were incubated for 6 h as described above. The inflammatory response and anti-inflammatory activity of the fermented products were determined by measuring the expression levels of inflammatory markers IL-1 β , IL-6, and TNF α , through real-time PCR gene expression. In particular, after incubation, cells were collected by trypsinization, washed once with PBS (Phosphate-Buffered Saline), and then lysed for RNA extraction.

The quantitative analysis was performed with One-Step Fast Real-Time PCR System Applied Biosystem using the SYBR Green PCR master mix (Life Technology, Milan, Italy). The primer sequences used are shown in Supplementary Table 2. The mix for PCR analyses included previously synthesized cDNA, SYBR Green PCR master mix (Life Technology, Milan, Italy), primer mix (forward primer/reverse primer), and UltraPureTM Distilled Water DNase/RNase Free (Invitrogen by Life Technologies, Milan, Italy). PCR reactions were subjected to 40 cycles of 95 °C for 20s, 95 °C for 3s, and 60 °C for 30s. The relative mRNA expression levels of each gene were determined by the threshold cycle (Ct) value of each PCR product and normalized with GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) by using comparative 2-DDCt method. The analysis was performed in duplicates.

6.3.12 Sensory evaluation of fermented products

The experimental fermented products (EY, EYGG, EYBB536, EYBB12, and EYM) were subjected to sensory evaluation by a panel

of 15 judges (7 females and 8 males, aged between 24 and 40 years) recruited among the staff of Council for Agricultural Research and Economics, Research Centre for Olive, Fruit and Citrus Crops, (CREA-OFA) Acireale, Italy. For selection and training “general guidelines for the selection, training and monitoring of selected assessors and expert sensory assessors” was used according to UNI EN ISO 8586:2014 rule. Judges were pre-screened based on (i) regular consumption of yogurt and non-dairy fermented products (at least once per month), and (ii) absence of taste/smell disorders. In addition, all panelists were informed about the possible presence of allergens (cereals containing gluten, peanuts, milk/dairy products, eggs, nuts, sesame, sulphites, and soybeans) in the experimental fermented sample. Fermented sample attributes were generated in consensus across eight 2-h sessions during which judges tasted an array of yogurts, fermented milk, and kefir. The sensory profile method (UNI EN ISO 13299:2016) was used for the sensory analysis. The standard UNI EN ISO provides guidelines on the global process of developing a sensory profile. Panelists were asked to evaluate the following descriptors: astringency, bitterness, sweetness, sourness, cereal-type flavor, fermented odor, creaminess, white color, off-odor, off-flavor, and overall acceptability, using a 10-point scale (ISO 4121: 2003), ranging from 1 (absence of the sensation or extremely dislike) to 10 (extremely intense or extremely pleasant). All evaluations were carried out in the morning, at the sensory laboratory of the CREA-OFA conforming to the UNI EN ISO 8589:2014 standard. The order of presentation was randomized among judges and sessions. Data were acquired by Smart Sensory box a direct computerized registration system (Smart Sensory Solution, Sassari, Italy).

6.3.13 Statistical analysis

Microbiological and chemical data were analysed by ANOVA (One way-Analysis of Variance) followed by Tukey's post-hoc test, in order to assess the overall differences between germinated brown rice samples (GBR1 and GBR2) and among experimental fermented products (EY, EYGG, EYBB536, EYBB12, and EYM). Statistical analysis was performed using XLSTAT PRO 5.7 (Addinsoft, New York, USA) and the reference level of significance was 0.05 in all the assays.

6.4 Results

6.4.1 Chemical composition and bioactive compounds detected in germinated brown rice

The mean value of pH, proximate composition, and bioactive compounds profile of germinated brown rice samples GBR1 and GBR2, obtained from three different germination batches (a, b, and c), are reported in Table 1. No statistically significant differences were recorded among the same type of sample (GBR1 or GBR2) derived from different germination batches (a, b, and c) (data not shown). The technological process had a negligible impact on fat, fiber, and ash content; GBR2 samples exhibited higher moisture and protein content than GBR1 samples, whereas carbohydrates and sugar were detected at higher concentrations in GBR1 samples (Table 1). Regarding bioactive compounds profile, GABA, butyric acid, phytic acid, and inositol were higher in GBR 2 than in GBR 1 samples, whereas oryzanol was detected at higher concentrations in GBR 1 samples.

6.4.2 Microbiological analysis of germinated brown rice samples

The mean values (expressed as log₁₀ cfu/ml) and standard deviations of the main microbial groups detected in GBR1 and GBR2

samples, obtained from three different germination batches (a, b, and c), are shown in Supplementary Table 3. Overall, yeasts and moulds, Enterobacteria, faecal coliforms, staphilococci, *E. coli*, *Listeria* spp., and *Salmonella* spp. were never detected in the analysed samples. In addition, within each GBR sample, no statistically significant differences were detected among the analysed germination batches (a, b, and c). Mesophilic aerobic bacteria were detected in all the analysed samples and the highest count was registered in GBR2 samples. Lactic acid bacteria and lactococci were found only in GBR2 samples with cell densities of about 5 log units and 2 log units, respectively (Supplementary Table 3). Data are reported as mean value and standard deviation of three determinations. Between germinated brown rice samples and among experimental fermented samples, different lowercase letters (a–b) in the same row, indicate a significant difference at $p < 0.05$ (ANOVA with Tukey's post-hoc test), respectively.

Table 1. pH, proximate composition (g/100 ml), energy (Kcal/100 g), and bioactive compounds (mg/ml) of germinated brown rice GBR1 and GBR2 samples, obtained from three different germination batches (a, b, and c), and of experimental fermented products (EY, EYGG, EYBB536, EYBB12, and EYM).

	Germinated brown rice samples		Fermented products				
	GBR1	GBR2	EY†	EYGG†	EYBB536†	EYBB12†	EYM†
pH	6.41 ^a ± 0.11	6.32 ^a ± 0.11	4.38 ^a ± 0.05	4.35 ^a ± 0.07	4.32 ^a ± 0.04	4.33 ^a ± 0.03	4.35 ^a ± 0.05
Proximate composition (g/100ml)							
Moisture	75.25 ^b ± 0.27	81.22 ^a ± 0.28	78.91 ^a ± 0.32	77.44 ^a ± 0.31	77.93 ^a ± 0.26	78.11 ^a ± 0.28	78.83 ^a ± 0.21
Protein	1.81 ^b ± 0.13	2.23 ^a ± 0.14	1.64 ^a ± 0.14	1.61 ^a ± 0.11	1.68 ^a ± 0.18	1.65 ^a ± 0.21	1.64 ^a ± 0.14
Fat	0.75 ^a ± 0.09	0.78 ^a ± 0.13	0.58 ^a ± 0.07	0.64 ^a ± 0.07	0.63 ^a ± 0.13	0.69 ^a ± 0.20	0.66 ^a ± 0.15
Fiber	0.64 ^a ± 0.24	0.71 ^a ± 0.28	0.72 ^a ± 0.34	0.75 ^a ± 0.20	0.76 ^a ± 0.12	0.78 ^a ± 0.26	0.70 ^a ± 0.18
Ash	0.35 ^a ± 0.06	0.36 ^a ± 0.05	0.33 ^a ± 0.02	0.32 ^a ± 0.18	0.35 ^a ± 0.09	0.38 ^a ± 0.08	0.31 ^a ± 0.09
Carbohydrates	20.42 ^a ± 0.41	14.79 ^b ± 0.38	17.82 ^a ± 0.49	17.86 ^a ± 0.26	17.73 ^a ± 0.24	17.80 ^a ± 0.26	14.78 ^a ± 0.21
Sugar	12.39 ^a ± 1.21	0.53 ^b ± 0.11	7.57 ^a ± 0.70	7.43 ^a ± 1.11	7.49 ^a ± 1.03	7.52 ^b ± 0.18	7.46 ^a ± 0.14
Energy (Kcal)	95 ^a ± 1.49	51 ^b ± 1.41	104 ^a ± 1.05	106 ^a ± 0.93	106 ^a ± 1.09	109 ^a ± 1.00	104 ^a ± 1.11
Bioactive compounds (mg/ml)							
GABA	178.7 ^b ± 4.47	227.0 ^a ± 4.80	359.0 ^a ± 8.0	35.9 ^a ± 1.5	63.9 ^a ± 1.6	112.0 ^c ± 0.3	149.0 ^b ± 0.7
Butyric acid	27.0 ^b ± 2.10	94.1 ^a ± 1.53	5.0 ^b ± 1.3	nd	nd	9.5 ^a ± 1.3	nd

Other activities

Phytic acid	0.8 ^b ± 1.67	73.7 ^a ± 1.77	nd	nd	nd	nd	1.8 ± 1.6
Inositol	27.3 ^b ± 2.03	58.5 ^a ± 2.23	18.1 ^c ± 1.9	18.1 ^c ± 1.3	12.3 ^d ± 1.4	22.9 ^b ± 0.9	31.5 ^a ± 0.8
Oryzanol	44.8 ^a ± 1.65	39.0 ^b ± 1.60	96.3 ^a ± 1.2	97.3 ^a ± 1.6	90.1 ^b ± 1.1	15.6 ^d ± 2.3	53.8 ^c ± 1.3

† EY: un-inoculated with probiotics; EYGG: inoculated with *Lactocaseibacillus rhamnosus* GG (ATCC 52103); EYBB536: inoculated with *Bifidobacterium longum* BB536 (ATCC BAA-999); EYBB12: inoculated with *Bifidobacterium animalis* subsp. *lactis* BB-12; EYM: inoculated with a mixture of the aforementioned probiotic strains. nd: below the detection limit.

Data are reported as mean value and standard deviation of three determinations. Between germinated brown rice samples and among experimental fermented samples, different lowercase letters (a–b) in the same row, indicate a significant difference at $p < 0.05$ (ANOVA with Tukey's post-hoc test), respectively.

6.4.3 Isolation and identification of autochthonous lactic acid bacteria

Eighty isolates, obtained from MRS and M17 agar plates, were considered LAB based on positive Gram reaction, nonmotility, absence of catalase activity and spore formation, and rod or coccial shape (Supplementary Table 4). The PCR-RFLP profiles of LAB isolates, in comparison to those obtained from reference strains, allowed to discriminate five clusters and to ascribe the isolates to the following species: *Lacticaseibacillus paracasei* (31), *Lacticaseibacillus rhamnosus* (26), *Limosilactobacillus fermentum* (11), *Lactococcus lactis* (7), and *Pediococcus pentosaceus* (5) (Supplementary Table 4). To confirm the species attribution, one representative member of each PCR-RFLP cluster was subjected to 16S rRNA gene sequencing. Details about Query cover (%), E-value, Identity (%), and Accession number of the sequenced strains (GBR 41, GBR 59, GBR 77, GBR 48, and GBR 7) are reported in Supplementary Table 4.

6.4.4 Acidifying and coagulation abilities of starter cultures

Fig. 1 shows the acidifying activity exhibited by both SST 31-SY 1 (inoculated in a 1:1, 1:2, and 2:1 ratio) and YoFlex starters. In detail, the YoFlex starter determined low acidification of the product. SST 31 and SY 1, combined in a 1:2 and 2:1 ratio, showed similar behavior determining a drop in pH value of 1.23 and 1.28, respectively. Differently, when SST 31 and SY 1 were combined in a 1:1 ratio, the starters showed higher acidifying activity reaching a pH of 4.38 after both 12 and 14 hours of incubation (Fig. 1). Based on the

visual evaluation of clot formation, no differences were observed about the coagulation ability exhibited by the SST 31 and SY 1 starters at 1:1, 1:2, and 2:1 ratio whereas YoFlex determined a poor clot formation.

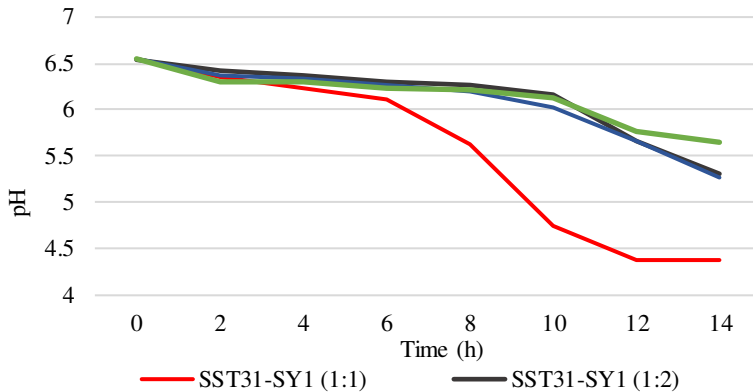


Fig. 1. Acidification profile during fermentation of germinated brown rice at 42°C by YoFlex and SST 31-SY 1 starters.

6.4.5 Microbiological analysis of experimental fermented products during storage

The microbiological stability of the experimental fermented products (EY, EYGG, EYBB536, EYBB12, and EYM), stored at +4 °C, as well as the viability of the adjunct probiotic strains, were weekly monitored for 28 days and results are reported in Table 2. Overall, yeasts and moulds, Enterobacteria and faecal coliforms, staphylococci, *E. coli*, *Listeria* spp., and *Salmonella* spp. were never detected in all the analysed samples. Total mesophilic bacteria and lactococci decreased during the storage time reaching, in the fourth week, values of about 6 log units and 2 log units, respectively. A high

count of lactobacilli and bifidobacteria was achieved, till the fourth week of storage under refrigerated conditions, in experimental fermented products inoculated with probiotics postulating their ability to survive during the shelf-life of the products.

Table 2. Microbiological stability of the experimental fermented products (EY, EYGG, EYBB536, EYBB12, and EYM) during storage at +4 °C for 28 days.

Microbial groups	Time (Weeks)	Experimental fermented products				
		EY [†]	EYGG [†]	EYBB536 [†]	EYBB12 [†]	EYM [†]
Mesophilic aerobic bacteria	1	7.15 ^a ±0.08	7.28 ^a ±0.05	7.17 ^a ±0.10	7.23 ^a ±0.07	7.21 ^a ±0.12
	2	7.03 ^a ±0.10	7.11 ^b ±0.08	7.07 ^a ±0.06	7.14 ^a ±0.02	7.09 ^b ±0.04
	3	6.74 ^b ±0.05	6.81 ^c ±0.06	6.83 ^b ±0.08	6.82 ^b ±0.05	6.78 ^c ±0.06
	4	6.51 ^c ±0.03	6.65 ^d ±0.09	6.59 ^c ±0.09	6.55 ^c ±0.10	6.63 ^c ±0.06
Lactobacilli	1	6.96 ^a ±0.06	9.94 ^a ±0.08	6.85 ^a ±0.05	6.76 ^a ±0.05	9.85 ^a ±0.07
	2	6.52 ^b ±0.08	9.46 ^b ±0.10	6.63 ^b ±0.03	6.78 ^a ±0.12	9.33 ^b ±0.05
	3	6.21 ^c ±0.03	9.34 ^c ±0.08	6.27 ^c ±0.06	6.64 ^b ±0.09	9.21 ^{bc} ±0.03
	4	6.03 ^d ±0.05	9.11 ^d ±0.06	6.08 ^d ±0.08	6.51 ^b ±0.06	9.14 ^c ±0.11
Bifidobacteria	1	nd	nd	9.85 ^a ±0.05	9.76 ^a ±0.05	9.77 ^a ±0.10
	2	nd	nd	9.63 ^b ±0.03	9.78 ^a ±0.12	9.19 ^b ±0.05
	3	nd	nd	9.27 ^c ±0.06	9.64 ^b ±0.09	9.11 ^{bc} ±0.03

4	nd	nd	9.08 ^d ±0.08	9.51 ^b ±0.06	9.00 ^c ±0.06
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† EY: un-inoculated with probiotics; EYGG: inoculated with *Lactiseibacillus rhamnosus* GG (ATCC 52103); EYBB536: inoculated with *Bifidobacterium longum* BB536 (ATCC BAA-999); EYBB12: inoculated with *Bifidobacterium animalis* subsp. *lactis* BB-12; EYM: inoculated with a mixture of the aforementioned probiotic strains.

nd: below the detection limit.

Data are reported as mean log value and standard deviation of three determinations. For each microbial group different lowercase letters (a–b) in the same column, indicate a significant difference among samples at $p < 0.05$ (ANOVA with Tukey's post-hoc test).

6.4.6 Chemical composition and bioactive compounds profile of experimental fermented products

Table 1 shows both the chemical composition and bioactive compounds profile of experimental fermented products EY, EYGG, EYBB536, EYBB12, and EYM. Overall, all the analysed samples showed similar proximate composition.

Regarding bioactive compounds profile, EY samples showed the highest concentration of both GABA and Oryzanol, whereas inositol was mainly detected in the EYM sample. Butyric acid was detected in both EY and EYBB12 samples, whereas the presence of phytic acid was revealed only in EYM sample.

6.4.7 Sensory evaluation of fermented products

Fig. 2 shows the sensory profiles of the experimental fermented products (EY, EYGG, EYBB536, EYBB12, and EYM) defined by fifteen trained judges. Sensory acceptability was high for all evaluated products. The sensory descriptors bitterness, fermented odour, and creaminess obtained similar scores in all samples. Astringency and sourness were mainly perceived in the EY sample whereas cereal-type flavour, white color, and sweetness descriptors were equally scored in EYGG, EYBB536, and EYBB12 samples. Samples EYGG and EYBB12 had the same ratings of sensory descriptors therefore their profiles overlapped. Off-flavour and off-odour were not perceived in any experimental fermented product.

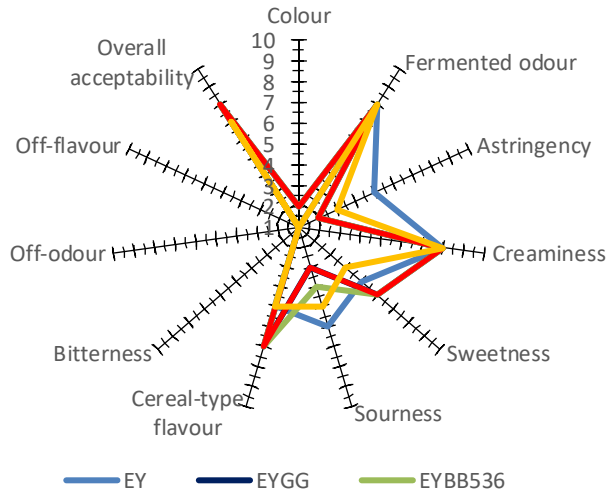


Fig. 2. Sensory Profile of the experimental fermented products (EY, EYGG, EYBB536, EYBB12, and EYM).

6.4.8 *Anti-inflammatory properties*

A human colon cell line, treated by LPS, was used as in vitro model to evaluate the anti-inflammatory activity of fermented products (EY, EYGG, EYBB536, EYBB12, and EYM). Fig. 3 shows that all fermented products did not induce significant changes in pro-inflammatory cytokines IL-1 β , IL-6, and TNF α gene expression. Fig. 4 shows that, in cells treated with LPS, there was an increase in pro-inflammatory genes compared to the control group. The co-treatment with EY, EYGG, EYBB12, and EYM inverted these harmful outcomes downregulating genes expression at values close to control.

Differently, the EYBB536 sample showed an increase in TNF α gene expression.

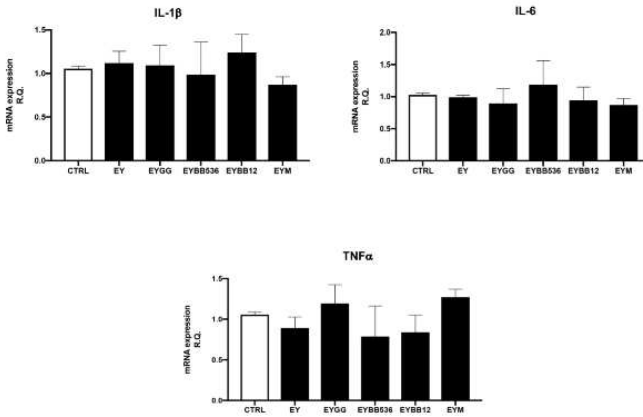


Fig. 3. Gene expression of interleukin-1 β (IL-1 β), interleukin-6 (IL6) and tumor necrosis factor- α (TNF- α) evaluated by RT-PCR. Bars represent the mean \pm SEM of six independent experiments.

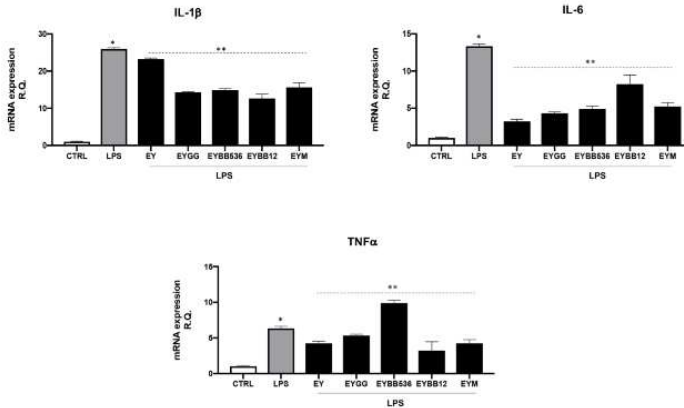


Fig. 4. Gene expression of interleukin-1 β (IL-1 β), interleukin-6 (IL6) and tumor necrosis factor- α (TNF- α) evaluated by RT-PCR. Bars represent the mean \pm SEM of six independent experiments. * $p < 0.05$ versus CTRL cells; ** $p < 0.05$ versus LPS treatment.

6.5 Discussion

In recent years, plant-derived foods and drinks, have gained more attention as valuable alternatives to animal-derived products. Consumer lifestyle choices and diet-related diseases (cardiovascular disease, elevated blood pressure, lactose intolerance, allergy to milk proteins) have led to higher demand, by both consumers and the food industry, for plant-based products (Montemurro, Pontonio, Coda, & Rizzello, 2021). In addition, these products are considered an economic and biotechnological choice to increase nutritional and functional features, along with the technological and sensory properties of non-dairy products (Coda, Montemurro, & Rizzello,

2017; FONA International, 2018).

In the present study germinated brown rice (GBR) fermented product functionalized with the addition of probiotics was formulated. GBR boasts peculiar nutritional properties thanks to the presence of bioactive molecules able to exert nutrigenomic effect. In particular, blood pressure and type II diabetes regulation, as well as the reduction in the risk of some chronic conditions, such as cancer, cardiovascular, and Alzheimer's diseases, have been demonstrated (Ravichanthiran et al., 2018; Wu, Yang, Toure, Jin, & Xu, 2013). Although, GBR is considered an interesting substrate, up to now few studies have been conducted to formulate novel GBR based-foods, such as bread (Cornejo, Cáceres, Martínez-Villaluenga, Rosell, & Frias, 2015), cookies (Chung, Cho, & Lim, 2014) and noodles (Gong et al., 2017). Only recently, Cáceres and co-workers (2019) investigated the suitability of different brown rice (BR) derivatives, such as BR flour, soaked and germinated BR, for the formulation of a yogurt-like product. However, to the best of our knowledge, the suitability of GBR for the formulation of yogurt-like brown rice products, functionalized with the addition of probiotics, has not been yet explored.

It is well known that the germination process enhances both the concentration and bio-availability of nutrients including γ -aminobutyric acid (GABA), lysine, vitamins, dietary fiber, niacin, magnesium, zinc, potassium, inositol, ferulic and phytic acids, tocotrienols, γ -oryzanol, and prolyl-endopeptidase inhibitor (Kim et al., 2020; Ravichanthiran et al., 2018; Patil & Khan, 2011). As well documented, the amount of bioactive compounds, accumulated during the germination process, vary greatly based on several factors such as pH, temperature, and time during soaking, germination, and post-germination (Cáceres, Martínez-Villaluenga, Amigo, & Frias 2014; Watchararparpaiboon, Laohakunjit, & Kerchoechuen, 2010). According to that, the highest amount of GABA, butyric acid, and

inositol detected in GBR2 samples is probably due to the germination process applied .

GBR is a food matrix rich in several nutrients that can be used as growth substrates by starters. In addition, its suitability for fermentation is related to the presence of both carbohydrates and sugar. In the present study, GBR1 samples exhibited higher concentration of both carbohydrate and sugar than GBR2 ones which could be related to the germination processes. In fact, alpha- and beta-amylase enzymes, used for the production of GBR1 samples, are involved in the transformation of starch into malts and simple sugars, determining an increase in both carbohydrates and sugar content. According to the fermentability of GBR, although differences among the tested starter cultures were observed, the mixture of the SST31 and SY1 strains showed an optimal acidification rate, in line with previously reported data (C´aceres et al., 2019; Chekdid et al., 2021; Nionelli et al., 2014). In fact, when the strains SST31 and SY1 were used in a 1:1 ratio, a fast drop in pH was detected. This was probably due to their rapid metabolism, the nature of the substrates consumed, and the incubation temperature. In addition, in spite of the low amount of proteins, a good coagulum was visualized without the need for structuring agents and emulsifier additions. The optimal texture, of the fermented products formulated in the present study, could be attributed to the ability of starters to synthesise exopolysaccharides (EPS), which was confirmed by the creaminess descriptor and acceptability by panelists. It is well known that EPS improve rheological properties and enhance sensory and mouth-feel characteristics of fermented products (Ripari, 2019). No off-flavours and off-odours were perceived by panelists and very low scores were attributed to bitterness and sourness, which are related to the possible occurrence of lipid oxidation during the germination process (Kince et al., 2017). Interestingly, in discordance to previously reported data

high perception of the fermented odour was revealed by pannelists (C´aceres et al., 2019; Chekdid et al., 2021).

In the present study, LAB strains were isolated from GBR samples and mainly genotypically ascribed to *Lacticaseibacillus paracasei*, *Lacticaseibacillus rhamnosus*, *Limosilactobacillus fermentum*, *Lactococcus lactis*, and *Pediococcus pentosaceus* species. The dominance of these species in plant-derived products is in accordance with other studies (Ziarno & Cicho´nska, 2021). The isolated strains were also characterized for technological features in order to formulate a good candidate as a starter culture. Based on the bioactive compounds profile of the formulated yogurt-like products, the present study highlighted that the fermentation process improved the nutritional value of GBR by increasing both GABA and oryzanol and by neutralizing the anti-nutritional phytic acid. In addition, both GABA and oryzanol contents increased in samples functionalized by the addition of probiotics indicating that GABA accumulation was initiated in the germination process and continued through both the fermentation and the *shelf-life* of the product, according to previous studies (C´aceres et al., 2014; C´aceres et al., 2019; Sen, Tewu, Lijun, & Shanbai, 2008). Notoriously, GABA, a bio-functional substance with several health-beneficial properties, is produced during the germination of brown rice (Cho & Lim, 2016; Ravichanthiran et al., 2018). The glutamate decarboxylase or the diamine oxidase of polyamines are responsible for GABA formation (Khwanchai, Chinprahast, Pichyangkura, & Chaiwanichsiri, 2014; Yang, Chen, & Gu, 2011). Although the GABA content change in relation to both rice variety and germination process parameters (e.g. pH, temperature, and time of soaking), the present study revealed higher GABA levels in both non-fermented and fermented products compared to previously published data (C´aceres et al., 2019; Chekdid et al., 2021; Cho & Lim, 2016). Interestingly, the fermentation process determined an increase

in the GABA content of >40%. This effect can be attributed to the metabolic activity explicated by the used starter cultures in line with previously reported evidence suggesting that, among bacteria, lactobacilli, including *Lactobacillus delbrueckii* strains, are considered the main producers of GABA (; Dhakal, Bajpai, & Baek, 2012; Seok et al., 2008; Siragusa et al., 2020; Yunes et al., 2016). Among nutrients, oryzanol is the most common sterol present in the bran of BR with documented physiological properties including antioxidant, anticarcinogenic, antihyperlipidemic, anti-inflammatory, and neuroprotective properties (Cicero & Gaddi, 2001; Francisqueti et al., 2017; Ravichanthiran et al., 2018). Our data revealed that the fermentation process determined a remarkable increase in oryzanol, in discordance with previously reported data (C´aceres et al., 2019; Chekdid et al., 2021), suggesting that the used starter stains could be able to synthesize oryzanol. This feature was previously demonstrated by Esteban-Torres, Rever´on, Manche˜no, de Las Rivas, and Mu˜noz (2013) for strains ascribed to the *Lactiplantibacillus plantarum* strains. In addition, our data revealed that, along with the enhancement of the bioactive compounds profile, the yogurt-like products, formulated in the present study, showed interesting anti-inflammatory properties on human colon cell line, treated by LPS, determining the reduction of proinflammatory cytokines IL-1 β , IL-6 and TNF α gene expression. Butyric acid was below the limit of detection in EYGG, EYBB536, and EYM samples. Its absence could be due to the ability of the used probiotic strains to metabolize butyric acid. The present study, investigated, for the first time, the suitability of GBR as a substrate for the formulation of fermented products functionalized by the addition of probiotic strains. As displayed by microbiological counts, all the fermented products, formulated in the present study, were stable and safe through the *shelf-life* of 28 days. In addition, based on the obtained results, both lactobacilli and bifidobacteria probiotic

strains added to the yogurt-like products were able to survive, at cell densities higher than 9 log units, during storage at +4 °C for 28 days. This evidence is not in line with previous studies indicating that the growth of *Bifidobacteria* spp. in cereal substrates is difficult unless a growth promoter (milk or yeast extract) is added (Gupta & Abu-Ghannam, 2012).

6.6 Conclusions

The present study demonstrated the suitability of GBR for the formulation of a plant derived fermented product. The fermentation process improved the nutritional composition of GBR, increasing the bioactive compounds content. In addition, high acceptability scores by panelists was recorded and the high level of lactobacilli and bifidobacteria indicated that GBR is a suitable matrix for the development of healthy plant derived foods.

Supplementary material

Supplementary Table 1. Reference strains used in the present study.

Species	DSM* code
<i>Enterococcus casseliflavus</i>	DSM 20680
<i>Enterococcus durans</i>	DSM 20633
<i>Enterococcus faecalis</i>	DSM 20478
<i>Enterococcus faecium</i>	DSM 20477
<i>Enterococcus gallinarum</i>	DSM 24841
<i>Enterococcus hirae</i>	DSM 20160
<i>Lactobacillus acidophilus</i>	DSM 20079
<i>Levilactobacillus brevis</i>	DSM 20054
<i>Lentilactobacillus buchneri</i>	DSM 20057
<i>Lacticaseibacillus casei</i>	DSM 20011
<i>Lactobacillus delbrueckii subsp. bulgaricus</i>	DSM 20081
<i>Lactobacillus delbrueckii subsp. lactis</i>	DSM 20072

<i>Limosilactobacillus fermentum</i>	DSM 20052
<i>Lactobacillus helveticus</i>	DSM 20075
<i>Lacticaseibacillus paracasei subsp. paracasei</i>	DSM 5622
<i>Lactiplantibacillus paraplantarum</i>	DSM 10667
<i>Lactiplantibacillus pentosus</i>	DSM 20314
<i>Lactiplantibacillus plantarum</i>	DSM 20246
<i>Lacticaseibacillus rhamnosus</i>	DSM 20021
<i>Latilactobacillus curvatus</i>	DSM 20019
<i>Lactococcus garviae</i>	DSM 20684
<i>Lactococcus lactis subsp. cremoris</i>	DSM 20069
<i>Lactococcus lactis subsp. lactis</i>	DSM 20481
<i>Lactococcus raffinolactis</i>	DSM 20443
<i>Leuconostoc citreum</i>	DSM 5577
<i>Leuconostoc lactis</i>	DSM 20202
<i>Leuconostoc mesenteroides</i>	DSM 20343
<i>Pediococcus acidilactici</i>	DSM 20284

<i>Pediococcus pentosaceus</i>	DSM 20336
<i>Streptococcus thermophilus</i>	DSM 20617

* DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH,
Braunschweig, Germany.

Supplementary Table 2. Primer pairs used in quantitative Real-Time PCR assay.

Gene	Forward Primer	Reverse Primer
α -SMA	GAGACCCTGT	TACATAGTGG
	TCCAGCCATC	TGCCCCCTGA
GAPDH	AGCTTCGGCA	CGTTCACTCC
	CATATTCATCTG	CATGACAAACA
IL-1 β	ATGATGGCTT	GTCGGAGATT
	ATTACAGTGGCAA	CGTAGCTGGA
IL-6	TTCCTCTCTGC	AGGAGAGCA
	AAGAGACTTCC	TTGGAAATTGGGG
TNF- α	AAGCACACTG	TGGGTCCTG
	GTTCCCACT	CATATCCGTT

Supplementary Table 3. Microbial counts expressed as average \log_{10} cfu/ml and standard deviation (SD) of the main microbial groups detected on GBR1 and GBR2 samples, obtained from three different germination batches (a, b, and c).

Microbial groups	Samples	
	GBR1	GBR2
Mesophilic aerobic bacteria	2.25 ^b ±0.08	6.64 ^a ±0.06
Lactic acid bacteria	nd	4.85 ±0.08
Lactococci	nd	2.25 ±0.07

nd: below the detection limit.

Data are reported as mean log value and standard deviation of three determinations. Different lowercase letters (a–b) in the same row, indicate a significant difference among GBR samples at $p < 0.05$ (ANOVA with Tukey's post-hoc test).

Supplementary Table 4. Phenotypic and molecular identification of lactic acid bacteria isolated from GBR2 samples

Isolates	Isolation medium	Morphology	PCR-RFLP profile and sequencing	Query cover (%)	E-value	Identity (%)	Accession number
GBR 9; GBR 10; GBR 16; GBR 26; GBR 36; GBR 41 ; GBR 42; GBR 43; GBR 53; GBR 58; GBR89	MRS	Rods	<i>Limosilactobacillus fermentum</i>	100	0.0	99.04	MT611892.1
GBR 1; GBR 3; GBR 17; GBR 24; GBR 20; GBR 22; GBR 23; GBR 28; GBR 37; GBR 44; GBR 45; GBR 47; GBR 50; GBR 54; GBR 57; GBR 59 ; GBR 60; GBR 61; GBR 64; GBR 65; GBR 67; GBR 68; GBR 73; GBR 75; GBR 79; GBR 82; GBR 85; GBR 86; GBR 87; GBR 88; GBR 90	MRS	Rods	<i>Lactocaseibacillus paracasei</i>	100	0.0	100	CP016355.1
GBR 6; GBR 5; GBR 12; GBR 13; GBR 14; GBR 21; GBR 25; GBR 27; GBR 29; GBR 31; GBR 32; GBR 33; GBR 35; GBR 39; GBR 40; GBR 46; GBR 49; GBR 51; GBR 52; GBR 55; GBR 56;	MRS	Rods	<i>Lactocaseibacillus rhamnosus</i>	100	0.0	100	CP086327.1

GBR 70; GBR 71; GBR 77 ; GBR 80; GBR 83							
GBR 2; GBR 8; GBR 11; GBR 18; GBR 19; GBR 34; GBR 48	M17	Cocci in chains	<i>Lactococcus lactis</i>	100	0.0	100	KJ477407.1
GBR 4; GBR 7 ; GBR 15; GBR 30; GBR 38	MRS		<i>Pediococcus pentosaceus</i>	100	0.0	99.86	CP028269.1

Isolates in bold were subjected to 16S rRNA sequencing.

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7 Other activities: Participation to conferences

Poster abstract presentation: 12th NIZO Dairy Conference Innovations in Dairy Ingredients (5 – 7 October 2021). Poster reference number P54: “Development of a kiwi-based vegetable milk coagulant”; **F.D. NICOSIA**, I. PUGLISI, R. LA CAVA, A. PINO, A. BAGLIERI, C. CAGGIA, C. L. RANDAZZO.

The growing interest in alternative milk-clotting sources is due to various factors such as the high price of calf rennet, religious concerns, vegetarianism or a ban on the use of recombinant calf rennet uses, especially in France, Germany and Netherlands. The most commonly used rennet substitutes include enzymes of microbial origin or plant extracts, proteases such as ficin from *Ficus* sp. latex, papain from *Carica papaya* and cardosins from *Cynara* sp. Among plant sources, kiwi fruit (*Actinidia chinensis*) contains high amounts of actinidin (EC. 3.4.22.14), a cysteine protease, which showed the highest potential for its use as a milk-clotting agent. In the present work a novel kiwi aqueous preparation was developed and used as coagulant enzyme in the manufacturing process of cheese. The extract was obtained from ripe and unripe kiwifruit and several tests were carried out in order to define the best performing concentrations to be used in dairy preparation. Furthermore, a polyacrylamide gel electrophoresis SDS-PAGE was performed, in order to determine the presence of the enzyme actinidin in the various tissues of the fruit and to evaluate the action towards k-casein and milk.

Data showed that an amount of kiwi extract corresponding to about 3% (v/v) results in a dairy yield of 20%, comparable to calf rennet yield. Results obtained from the SDS-PAGE revealed presence

of the actinidin both in the fruit peel and in the pulp. However, the aqueous extract obtained from the kiwi peel does not seem to be able to break down the k-casein, while the aqueous extract obtained from the pulp showed an hydrolytic activity comparable to that of chymosin towards k-casein and milk. Due to its properties as well as to the accessibility of the primary source for the preparation of the aqueous solution, kiwi fruit represents a good candidate as milk clotting substitution.

Poster abstract presentation: Microbial Diversity 2021, Advances in Microbial Diversity (December 14-15, 2021). Title: “Novel vegetable coagulant extracted from kiwifruit”; **F.D. NICOSIA**, I. PUGLISI, R. LA CAVA, A. PINO, A. BAGLIERI, C. CAGGIA, C. L. RANDAZZO

The worldwide increase in cheese production, alongside with the reduced supply of calf rennet, higher prices, religious reasons and diet such as vegetarianism, have led to an increase in the demand for alternative sources of milk coagulants. Enzymes extracted from plants, proteases like ficin from *Ficus* sp. latex, papain from *Carica papaya* and cardosins from *Cynara* sp. have become a subject of growing interest in dairy technology. Unfortunately, many of these enzymes produce extremely bitter compounds, reaching unpleasant the final product. In the present study the preparation of kiwifruit (*Actinidia chinensis*) extract proteins was carried out, and the clotting activities of kiwifruit extracts were determined by using aqueous extract from pulp and peel from both ripe and unripe fruits. In addition, the composition of coagulum by the milk clotting action of the different kiwifruit extracts was analysed by a polyacrylamide gel electrophoresis SDS-PAGE, and patterns were compared with that obtained by calf chymosin action. Subsequently, kiwifruit’s aqueous

extracts were used as coagulant for a laboratory scale cheesemaking. Results obtained from the SDS-PAGE revealed the presence of the actinidin both in the fruit peel and in the pulp of both ripe and unripe fruits. The kiwifruit extract from the pulp showed a hydrolysis patterns towards k-casein and milk similar to that produced by chymosin, while the extract from the peel does not seem to be able to hydrolyse k-casein. Laboratory scale cheesemaking confirmed the low clotting activity of kiwifruit extract from peel, while a comparable cheese yield was achieved by aqueous extract obtained from ripe kiwifruit's pulp. Due to its qualities, the kiwifruit's extract from pulp is a promising substitute to animal rennet, for the easy availability of the raw material by using overripe or undersize fruits in a circular economy perspective.

Poster abstract presentation: “Sicilian canestrato cheese produced with kiwifruit extract”; LUIGI LIOTTA, FABRIZIO NICOSIA, FEDERICA LITRENTA, VINCENZO LOPREIATO, CARMELO CAVALLO, ROSITA LA CAVA, MARCO SCALISI, CINZIA CAGGIA AND CINZIA RANDAZZO. 75°Convegno SISVET. June 15th-18th, 2022.

The worldwide increase in cheese production, combined with the reduction in supply and religious reasons (Islam and Judaism), have led to the search for alternative enzymes for coagulation of milk, as appropriate substitutes of animal rennet. Enzymes extracted from plants, proteases like ficin from *Ficus* sp. latex, papain from *Carica papaya* and cardosins from *Cynara* sp. have become a subject of growing interest in dairy technology. Among plant coagulants, kiwifruit (*Actinidia deliciosa*) contains high amounts of actinidin (EC. 3.4.22.14), a cysteine protease, which showed a high potential for its use as a milk-clotting agent in cheese making process. The aim of the

present study was to evaluate the effect of kiwifruit enzyme on the quality of Sicilian Canestrato cheese. In addition, the quality parameters were compared to those of Canestrato cheese made with commercial lamb rennet (Caglio pasta provo 5, Caglificio Clerici, Italy). In detail, pasteurized milk was clotted by kiwifruit (*Actinidia deliciosa*) extract (3% v/v) following the traditional flowchart for the Canestrato cheese production. The curd was broken into small granules with a thorn, and then pressed, to facilitate the purging of the whey, then brined and stored at 14°C and 70-80% humidity at least for 15 days. The pasteurized milk and the resulting cheese were subjected to chemical determinations. Moreover, total polyphenols content of experimental and commercial Canestrato cheese samples was determined spectrophotometrically using Folin-Ciocalteu's reagent. Results showed that no significant differences were observed for total lipids (CONTROL 20.54±0.51% vs CHEESHAL 19.97±0.16%), total proteins (CONTROL 24.44±0.25% vs CHEESHAL 24.80±0.33%), while significant differences were recorded for the polyphenols content (mg/100g of cheese: CONTROL 88.02±0.50 vs CHEESHAL 141.07±0.84; P<0.05). Cheese made with kiwi extract showed a lower cheese yield (13%) than cheese from lamb rennet (16%). The preliminary results of this study show the possibility to use kiwi extract as a promising alternative to commercial rennet in milk coagulation, suggesting that the kiwifruit extract is a potential vegetable source of coagulant for the manufacturing of Sicilian Canestrato cheese with improved nutritional characteristics. Study funded by PSR SICILIA 2014/2020. Misura 16, Sottomisura 16.1, CHEESHAL. CUP G66D20000640009.

Poster abstract presentation: “Technological Characterization Of Italian And Brazilian Lactic Acid Bacteria Strains For Potential Use In Cheese Manufacture”; Andressa Fusieger (1);

Fabrizio Domenico Nicosia (2); Alessandra Pino (2); Guilherme Lembi Ramalho Maciel (1); Rosamaria Roberta Sanfilippo (2); Cinzia Caggia (2); Antonio Fernandes De Carvalho (1); Cinzia Randazzo (2). Simposion internacional ABRAPA. San Paolo March 15th-17th 2022.

Lactic acid bacteria (LAB), normally present in milk, utensils, and surfaces of dairy farms, play an important role in cheesemaking, both in the early stages of milk fermentation and during cheese ripening. The aim of this work was to characterize the technological features of LAB strains, isolated from different Italian and Brazilian traditional cheeses, in order to set up an adjunct culture for cheesemaking. A total of 26 LAB isolates were tested for their use in cheesemaking, being previously identified as follow: *Lactiplantibacillus plantarum* Q1C6, Q3C4, Q5C9 and Q6C4 (n=4); *Pediococcus acidilactici* Q1C8, Q3C1, Q3C3, Q6C1, Q6C5 and Q22C2 (n=6); *Lactococcus lactis* subsp. *lactis* Q5C6 (n=1); *Lactobacillus delbrueckii* P7, P9, P10, P11, P12, P13, P14, P15, P33, P36, P37, P38, P39 and P40 (n=14) and *Lacticaseibacillus rhamnosus* P50 (n=1). Isolates were screened for salt tolerance, exopolysaccharide and diacetyl production, lipolytic, acidifying and proteolytic activities. In addition, the aminopeptidase (Pep N and Pep X) activities, were evaluated. Overall, all the tested strains, with the exception of two *L. plantarum* strains (Q1C6 and Q3C4), showed low or medium acidifying activity and proteolytic activity. Lipolytic activity was revealed in none of the strains, while the production of EPS and diacetyl was widespread and variable among the tested strains. Furthermore, most of the strains demonstrated salt tolerance to 6% of NaCl, while only two *L. delbrueckii* (P14, P38), one *L. rhamnosus* (P50) and one *L. plantarum* (Q3C4) were able to grow in the presence of 10% (w/v) of NaCl. Finally, regarding aminopeptidase activities, 1 *L. delbrueckii* (P10), 1 *L. rhamnosus* (P50) and 1 *L. lactis*

(Q5C6) were considered as the better performing, showing high values of both Pep N and Pep X. Based on data, the salt resistance, the low acidifying and lipolytic activities, the ability to produce diacetyl and EPS as well as the aminopeptidase activities allowed us to indicate the strains Q5C6, P10, and P50, ascribed to *L. lactis*, *L. delbrueckii* and *L. rhamnosus*, respectively, as the most promising strains to be used as adjunct cultures. In addition, the high aminopeptidase activity produced by the aforementioned strains could help to enhance the sensory properties of cheese, improving the overall quality of the final product.

Poster abstract presentation: 7th International Conference on Microbial Diversity 2023, Parma, 26th – 29th September 2023

Use of *Lactococcus lactis* Q5C6 strain as debitter adjunct culture for cheese clotted with kiwifruit enzymatic extract

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The use of plant-based milk-clotting enzymes in cheesemaking allows to obtain cheeses suitable for vegetarian diet and compliant with the Halal/Kosher standards. The only limitation is given by the high proteolytic activity of these enzymes, which lead to the production of short bitter peptides that alter both cheese flavor and texture. However, by using specific bacterial aminopeptidases (aminopeptidases N and X) is possible to further hydrolyze these short peptides, reducing bitter taste in cheese. In the present work the debittering activity of a *Lactococcus lactis* (Q5C6) strain was investigated: the latter was used as adjunct culture in cheese clotted with kiwifruit enzymatic extract to improve the sensory profile of the final product. In detail, the optimal amount of kiwifruit enzymatic extract, to be used in cheesemaking, was determined by performing laboratory-scale coagulation tests. Furthermore, two experimental cheesemaking trials (A and B) were carried out and the obtained cheeses were subjected to physico-chemical, microbiological, and sensory analysis. Results of laboratory-scale coagulation tests show that the use of 0.7 g/l of kiwifruit enzymatic extract allowed to obtain acceptable coagulation time and slight bitter taste. Data revealed that the use of the kiwifruit enzymatic extract determined changes in fat, ash, and protein content in the final cheeses compared to cheese clotted through the animal rennet. Moreover, the *Lactococcus lactis* Q5C6 strain, used as adjunct culture at 1% (v/v), decreased the bitter taste, generating a cheese with a sensory profile comparable to cheese clotted by animal rennet. In conclusion, *Lactococcus lactis* Q5C6 is a promising strain in cheese debittering, produced with kiwifruit clotting enzyme.

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Abstract presentation: 5th Joint workshop of the agriculture-oriented PhD programs at Unict, Unifg and Uniud”. September 25th-28th 2023. Monastero dei Benedettini, Catania (CT)

Experimental cheesemaking using plant-based milk-clotting enzyme and debittering adjunct culture

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In the present study a kiwifruit enzymatic extract was used as milk-clotting enzyme for cheesemaking. The presence of the enzyme actinidin in the tissues of the kiwifruit was achieved through a polyacrylamide gel electrophoresis SDS-PAGE. In addition, the *Lactococcus lactis* subsp. *lactis* bv. *diacetylactis* Q5C6 strain, selected for specific aminopeptidase activity, was used as adjunct culture to reduce the bitter compounds generated by actinidin, during cheese manufacture and ripening. Two experimental cheesemaking trials were carried out and the obtained cheeses were subjected to physico-chemical, microbiological and sensory evaluation and compared to cheese clotted with animal rennet, used as control. Results showed that, the use of the kiwifruit enzymatic extract determined changes in fat, ash, and protein content respect to control cheese. In addition, higher values of mineral compounds were observed in the experimental cheeses than control ones. The low protein content observed in cheese samples clotted by using the kiwifruit extract and inoculated with the debittering adjunct culture could be related to the proteolytic activity exerted by the selected adjunct culture. Interestingly, the *L. lactis* subsp. *lactis* bv. *diacetylactis* Q5C6 strain,

inoculated at 1% (v/v), was able to reduce the bitter taste obtaining a final product with a sensory profile comparable to cheese clotted by animal rennet.

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