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Technological and probiotic related assessment of new Lactic Acid Bacteria isolated from traditional fermented foods by physiological and molecular approaches

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Contents

Abstract

Fermentation is an old technology to preserve foods for a longer time and to prepare different foodstuffs for local consumption. Fermented foods have had a long history in different countries due to homemade processing. Different types of traditional fermented foods and beverages are produced all over the world. Interestingly, in traditional fermentation process, commercial starter microbes are not used, and it is usually common to use the previous homemade product as a natural starter. Therefore, the community of microorganisms present can be pretty unknown and very interesting to be studied. Fermented foods are complex microbial ecosystems; many of them contain Lactic Acid Bacteria (LAB) as the predominant group playing a significant role in the fermentation process. Food fermentation by LAB usually provides palatability, high sensory quality, texture, stability, and nutritional properties. On the other side, potentially probiotic LAB, which can prevent or treat some illnesses and benefits human health, such as cholesterol-lowering capability, treatment or prevention of diarrhea, vitamin production and anti-cancer activity, are even more attractive bacteria to be further studied. In this thesis, we have evaluated the technological and probiotic-related properties of new lactic acid bacteria isolated from traditional fermented foods by physiological and molecular approaches.

Firstly, we have studied the microbial diversity and nutritional properties of "Yellow Curd" (*Kashk zard*) a traditional Persian fermented food, using classical microbiology approaches and 16S rRNA metabarcoding analysis. The metagenomics analysis revealed that lactic acid bacteria, particularly the genera *Lactobacillus, Pediococcus* and *Streptococcus*, were dominant in Yellow Curd. In addition, isolation, characterization and identification of new LAB with technological potential and probiotic properties, as well as functional foods preparation and characterization, have been carried out. The outcomes revealed that *Pediococcus acidilactici* strains IRZ12A and IRZ12B isolated from traditional Persian fermented food were safe and endowed with probiotic properties such as resistance to simulated gastrointestinal conditions and hypocholesterolemic effect. The complete genome of this promising *P. acidilactici* IRZ12B strain with the best cholesterol-lowering effect was sequenced, and the *in-silico* analysis evidenced the absence of plasmids, transmissible antibiotic resistance genes and virulence factors. Therefore, we tried to use *P. acidilactici* IRZ12B and three officinal plants, namely *Malva, Calendula* and *Echinacea*, for fermentation trials and consequently, possible functional food production. The fermented herbs with *P. acidilactici* IRZ12B were used to study their functionality on healthy and cancer cells.

Results showed that fermented herbs with the probiotic strain did not have any cytotoxic effect on healthy human intestinal cells (differentiated Caco-2); however, they could significantly reduce cell viability on breast cancer cells (MCF-7).

Secondly, some beneficial strains did not tolerate the storage condition and the human gastrointestinal passage. In this thesis, for the first time, we evaluated the use of 2′-fucosyllactose molecule in microencapsulation, and compared it with other known molecules, such as gelatin and inulin. Microcapsules, obtained by the extrusion technique, were evaluated in terms of encapsulation efficiency, storage stability, gastrointestinal condition resistance, and cell release kinetics. The alginate-based microcapsules showed interesting features, including the good capability to protect bacterial cells from harsh simulated gastrointestinal conditions. Compared to other molecules used in microencapsulation together with alginate, such as gelatin and inulin, 2'-fucosyllactose evidenced an extremely quick and abundant release of bacterial cells from the capsules inside a simulated intestinal fluid.

Lastly, we have studied the technological properties of LAB isolated from a traditional fermented Indian food (*Dahi*). As a result, strain *Limosilactobacillus fermentum* ING8 was found to possess a safe status with very interesting technological properties, such as galactose utilization, antimicrobial activity, exopolysaccharide production and survivability to long-term storage at refrigeration temperature. The complete genome evaluation of this promising strain confirmed the absence of possible deleterious elements, such as acquired antibiotic resistance genes, virulence genes, or hemolytic-related genes. However, all structural genes related to galactose operon and EPS production have been detected.

Chapter 1

Microbial diversity and nutritional properties of Persian "Yellow Curd" (*Kashk zard*), a promising functional fermented food

Abstract

"Yellow Curd" (YC) is one of the most popular homemade Persian fermented foods and is consumed by many people. Notwithstanding, no studies are available to date on its nutritional and microbiological composition. In this study, we examined YC samples obtained from different local markets of Sistan and Baluchestan province, Iran. The results of the chemical analyses revealed a homogenous content of protein (13.71% ± 1.07), lipids (4.09% ± 0.73), and carbohydrates (61% ± 2.13) among the samples. By comparing the average mineral content of YC with yogurt, many relevant differences were detected. Apart from the calcium content, which was similar on average to that of YC, all other minerals tested are present in higher amounts in YC than in yogurt. The analysis of the main sugars present (i.e., lactose, galactose and glucose) highlighted relevant differences among samples, indicating that different YC samples contain natural strains with different capabilities to metabolize sugars. The concentration of galactose in YC samples should be taken into consideration by galactose intolerant people. From the microbiological perspective, the metagenomics analysis revealed that lactic acid bacteria, and particularly the genera *Lactobacillus*, *Pediococcus*, and *Streptococcus,* were dominant in YC. The information provided shows that YC is an interesting base for the preparation of novel functional foods with a good content of beneficial bacteria.

1.1. Introduction

Iran is a country rich in traditional foods that come from an ancient food preparation history.

"Yellow Curd" (YC) is one of the most popular homemade food products in the south-eastern part of Iran (i.e., Sistan and Baluchestan provinces), but it is also largely produced and consumed in other parts of the country, including the Khorasan province and the capital, Tehran.

Generally, YC is prepared by a combination of homemade yogurt (made from cow milk), wheat flour, different types of local aromatic herbs and spices (dill, coriander, cumin, turmeric, garlic) and salt. The preparation process includes two fermentation steps. First, a dough is made by adding wheat flour to the yogurt and maintaining the resulting mixture inside a specific earthen container for two weeks in a dark and warm (around 30 °C) place. Then, fresh yogurt and seasonings are added, kneaded with the dough and the resulting mix is again stored at the same conditions for another 7 to 10 days, during which a second fermentation takes place. The product is then distributed outside on the surface of sheets of textile and left to dry for up to 10 days. Lastly, the dried YC is ground to get a granulated product of 1–3 mm sized particles. YC is

consumed as a soup, prepared by dissolving about 6 g of powder in 1 L of water. To date, no studies are available in the literature on the chemical and microbiological characteristics of this product. Indeed, information about YC composition would be important to determine its nutritional value and the possible influence of food components on microbial selection and development. Knowledge of its microbiota composition would be relevant, since it is ascertained that microbes considerably influence many characteristics of fermented products, including safety, sensory characteristics, and nutritional aspects. To our knowledge, this study is the first report on the microbiological and chemical characteristics of YC, a poorly studied traditional product which constitutes one of the most consumed daily meals by people in several regions of Iran.

1.2. Materials and Methods

1.2.1. Sample collection and processing

A total of 13 "Yellow Curd" (YC) samples were collected from seven different local household traditional markets in the Sistan and Baluchestan provinces (from different cities) in the south-east of Iran during August 2018 (Figure 1.1). Samples (approximately 100 g each) were collected in sterile plastic tubes, which were tightly capped and stored at room temperature. Samples were named from IRZ1 to IRZ13.

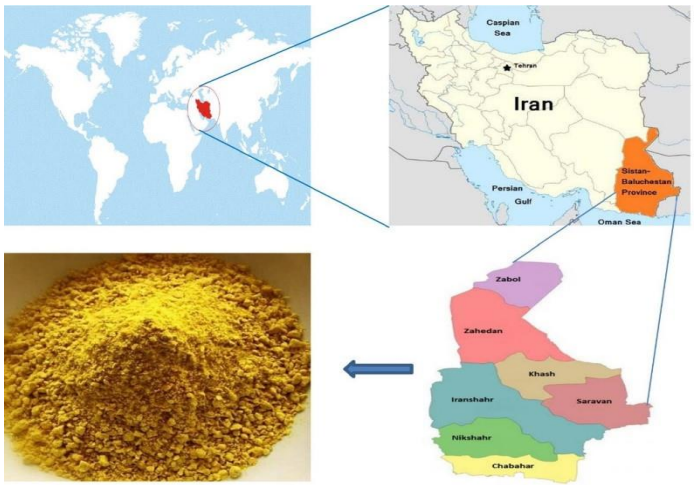

Figure 1.1. Geographic location of the Sistan and Baluchistan provinces in Iran and image of "Yellow Curd" (YC) powder.

1.2.2. pH measurement

pH determination of YC samples was performed according to Zaika et al. (1) with slight modification. Briefly, 20 g of YC samples were added to 160 mL of distilled water and mixed thoroughly. The solution was filtered, and 50 mL of this solution was further diluted 1:1 with 50 ml of distilled water. The pH was measured using a digital pH meter (Orion Star A211, Thermo Fisher Scientific, Waltham, MA, USA) by immersing the electrode into the solution.

1.2.3. 16S rRNA gene amplicon target sequencing

Total genomic DNA was extracted using the DNeasy PowerSoil Microbial Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The quality and quantity of the extracted DNA were assessed by 1% agarose gel electrophoresis and with a Spark 10M spectrophotometer (Tecan Trading AG, Männedorf, Switzerland), respectively. The V3–V5 regions of the 16S rRNA genes were amplified by PCR and sequenced using an Illumina MiSeq desktop sequencer (Eurofins Genomics Germany GmbH, Ebersberg, Germany), producing 300 bp paired-end (PE) reads. Three separate aliquots (technical replicates) were analyzed for each YC sample. Then, 16S rRNA sequences were analyzed using the software CLC Genomics Workbench (V.8.0.2) with the microbial genomics module plugin (QIAGEN Bioinformatics, Germany). Finally, the Operational Taxonomic Units (OTUs) that were not automatically attributed were manually assigned to the genus level by similarity, using BLASTN Megablast. Raw reads were deposited in the Sequence Read Archive (SRA) database (10.1093/nar/gkq1019).

1.2.4. Chemical and mineral compositions of YC samples

Grinding of YC samples was done using a GRINDOMIX GM200 Retsch homogenizer for 10 sec at 4500 rpm. Protein and lipid content, as well as ash and dry matter weights, were determined as described by William (2).Minerals (namely calcium (Ca), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), phosphorus (P), and zinc (Zn)) were measured as described by(3), using a Milestone Start microwave oven (Milestone Srl Sorisole Bergamo, Italy) and the results were collected using a Inductively Coupled Plasma Optical Emission Spectrometry (ICP-

OES) Spectro Arcos (Spectro Analytical Instruments GmbH, Kleve, Germany). Each analysis was performed in triplicate.

1.2.5. Quantification of sugar content

Lactose, glucose, and galactose quantification in all samples was performed in triplicate by high performance liquid chromatography (HPLC). All solvents were filtered using 0.45 μm filters. Ultrapure water was used for all dilutions and to prepare lactose, glucose and galactose standard solutions at concentrations between 0.005% (0.05 g/L) and 0.25% (2.5 g/L). Briefly, 5 g of YC samples were mixed with 50 mL of 7% perchloric acid (HClO4) in a volumetric flask and agitated for 10 min to precipitate proteins. Then, the pH was adjusted to 7.0 with 0.1 N NaOH and the solution was centrifuged at 6000 rpm for 2 min. Then, 1 mL of each sample was filtered using 0.22 μm Polytetrafluoroethylene (PTFE) filters. Aliquots of 20 μL were then injected into the HPLC for the analysis using a HyperRez XP Carbohydrate Ca2+ (300 × 7.7 mm) column and the following parameters: mobile phase = 100% ultrapure water, flow rate = 0.6 mL/min, pressure = 1100 psi, oven temperature = 80 °C, detection = RI(Refractive Index). The HPLC analysis time was 8:8 (min:sec) for lactose, 10:4 (min:sec) for glucose, and 11:25 (min:sec) for galactose, respectively.

1.2.6. Statistical analysis

Data collected from chemical and mineral analyses were evaluated by one-way analysis of variance (ANOVA) using Minitab software (version 19, Minitab software, PA, USA).

The relative abundance of each OTU for every YC sample obtained from the bioinformatics analyses was normalized by log transformation log10 (xi + 1). Bacterial similarity among samples was evaluated by ANalysis Of SIMilarity (ANOSIM) with 9999 permutations using the Bray–Curtis similarity index in the PAleontological STatistics (PAST) 3.26 (4) software. The Statistical Analysis of Metagenomics Profiles (STAMP) software (5) was used to plot principal component (PC) diagrams, as well as to identify significant differences in terms of genus among different samples using ANOVA. Tukey–Kramer ($p < 0.05$) was chosen as the post-hoc test and Bonferroni was used for multiple test corrections. Alpha diversity indexes were compared within all YC samples using ANOVA in GraphPad Prism 7 (GraphPad Software LLC, La Jolla, CA, USA). Tukey test ($p < 0.05$) was used to adjust for multiple comparisons.

1.3. Results and Discussion

1.3.1. 16. S rRNA Gene amplicon target sequencing

In this study, we analyzed 12 samples of YC with three technical replicates each. By conducting a high throughput amplicon sequencing of the hyper variable regions V3–V5 of the 16S rRNA gene, a total of 943,135 high-quality sequences with an average length of 254 bp were obtained after the removal of low quality and chimeric sequences. Unfortunately, due to the excessively low number (less than 1000) of reads obtained, sample IRZ1 was excluded from the analyses, which then included 12 samples (from IRZ2 to IRZ13). The procedure adopted for DNA extraction did not reasonably determine the lysis of bacterial endospores; therefore, such microbes are not considered in the genetic analysis. Regarding the analysis of alpha diversity. The Chao1 and Shannon indices, accounting for the number of different species (richness) and microbial distribution (evenness), respectively, varied significantly among the samples ($p < 0.05$) (Figure 1.2).

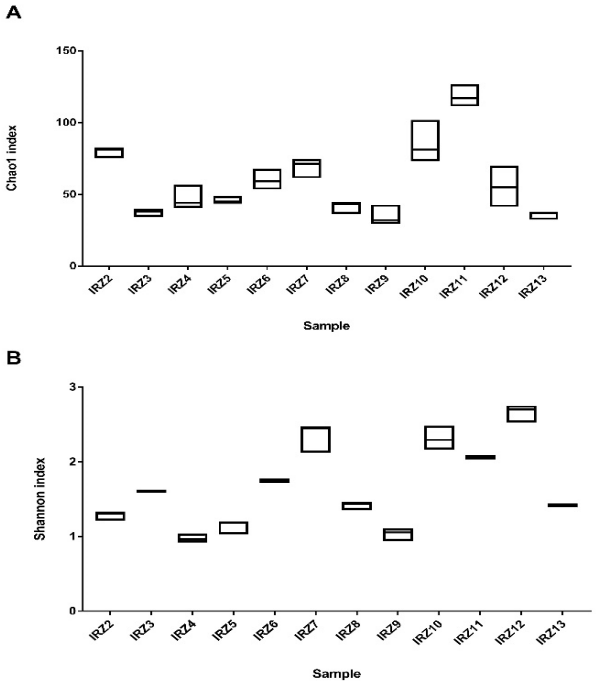

Figure 1.2. Alpha diversity: Chao1 (**A**) and Shannon (**B**) analyses regarding the number of species (richness) and microbial distribution (evenness), respectively, across all samples ($p < 0.05$).

Indeed, multivariate analysis (ANOSIM, p = 0.0001, ANOSIM statistic R (R) = 0.9976) indicates a highly dissimilar microbial composition among YC samples (Figure1.3), showing a relevant local influence on the bacterial diversity of these homemade products. Overall, principal components 1, 2 and 3 explain more than 93% of the variability (Figure1.4).

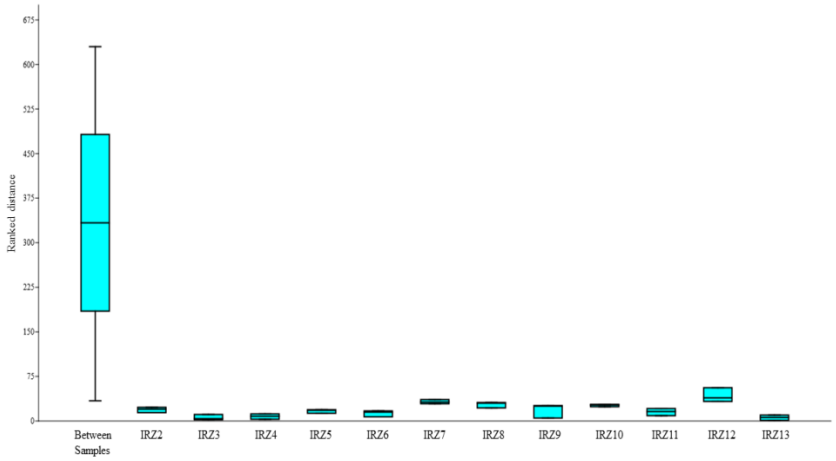

Figure 1.3. Boxplot of ranked distance obtained in the ANOSIM test between groups and for twelve Yellow Curd samples obtained from different places in Iran.

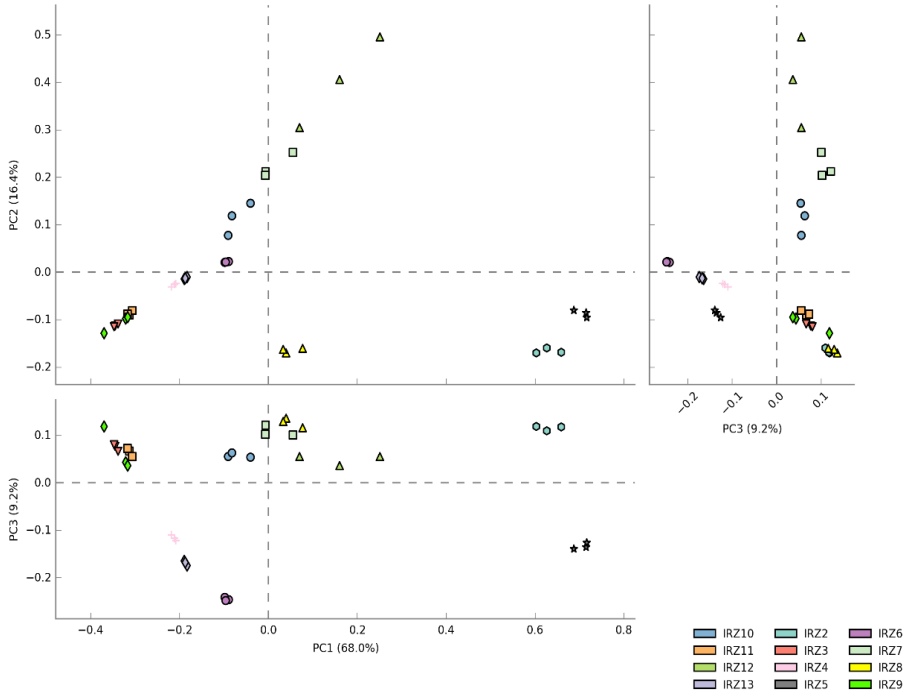

Figure 1.4. Plot of the three principal components (PC) determined after principal component analysis (PCA) of 16S rRNA data from twelve Yellow Curd samples. Technical replicates from the same Yellow Curd are represented by equal symbols with the same color.

After the clustering process (at a 97% similarity threshold), a total of 327 OTUs were identified. In total, 71 OTUs with more than 0.5% relative abundance were considered as "the most abundant". In regard to taxonomy, it was possible to assign 76% of the sequences at the phylum level, 59% at the genus level, and 16% at the species level. At the phylum level, *Firmicutes* (92%) were highly predominant, followed by *Proteobacteria* (4%) (Figure 1.5A). This is in accordance with published studies [6,7] that reported that the bacterial microbiota of naturally fermented milk includes *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes,* but *Firmicutes* represent the large majority. Interestingly, the phylum *Bacteroidetes* was not included among the most abundant OTUs, probably as a consequence of the high sensitivity of these bacteria to oxygen present during YC production and storage (8); it could also be considered an index of good hygienic environmental level, since *Bacteroidetes* are mainly bacteria of intestinal origin. Additionally, the significant presence of *Cyanobacteria* could reasonably be attributed to chloroplast DNA from plant material (i.e., vegetables and seasonings) that are added to the curd.
At the genus level, *Lactobacillus* (57%), *Pediococcus* (14%) and *Streptococcus* (12%) were the most abundant (Figure1.5B). Members of the genus *Lactobacillus* have a relevant role in the production of traditional fermented foods, since this heterogeneous genus includes thermophilic species able to grow well by fermenting several sugars (9, 10) .Of the bacterial sequences assigned to *Lactobacillus* species (*L. brevis*, *L. plantarum*, *L. reuteri*, *L. vaginalis*, and *L. zeae*), *L. plantarum* was identified in all samples in this study (Spreadsheet 1). As reported by several studies, this species is frequently isolated from traditional dairy products and contains strains with remarkable probiotic and technological properties(11, 12). We also conducted a MegaBLAST search to find the best hit at species level for the OTUs assigned to the genus *Lactobacillus*. Interestingly, this analysis allowed for the identification of three other species, namely *L. delbrueckii*, *L. pontis* and *L. fermentum*, which are frequently isolated from sourdoughs (naturally fermented flour–water mixtures used in the production of baked goods). These microbes could come from cereals that are added to YC in the manufacturing process, which is a potential source of lactobacilli (13).

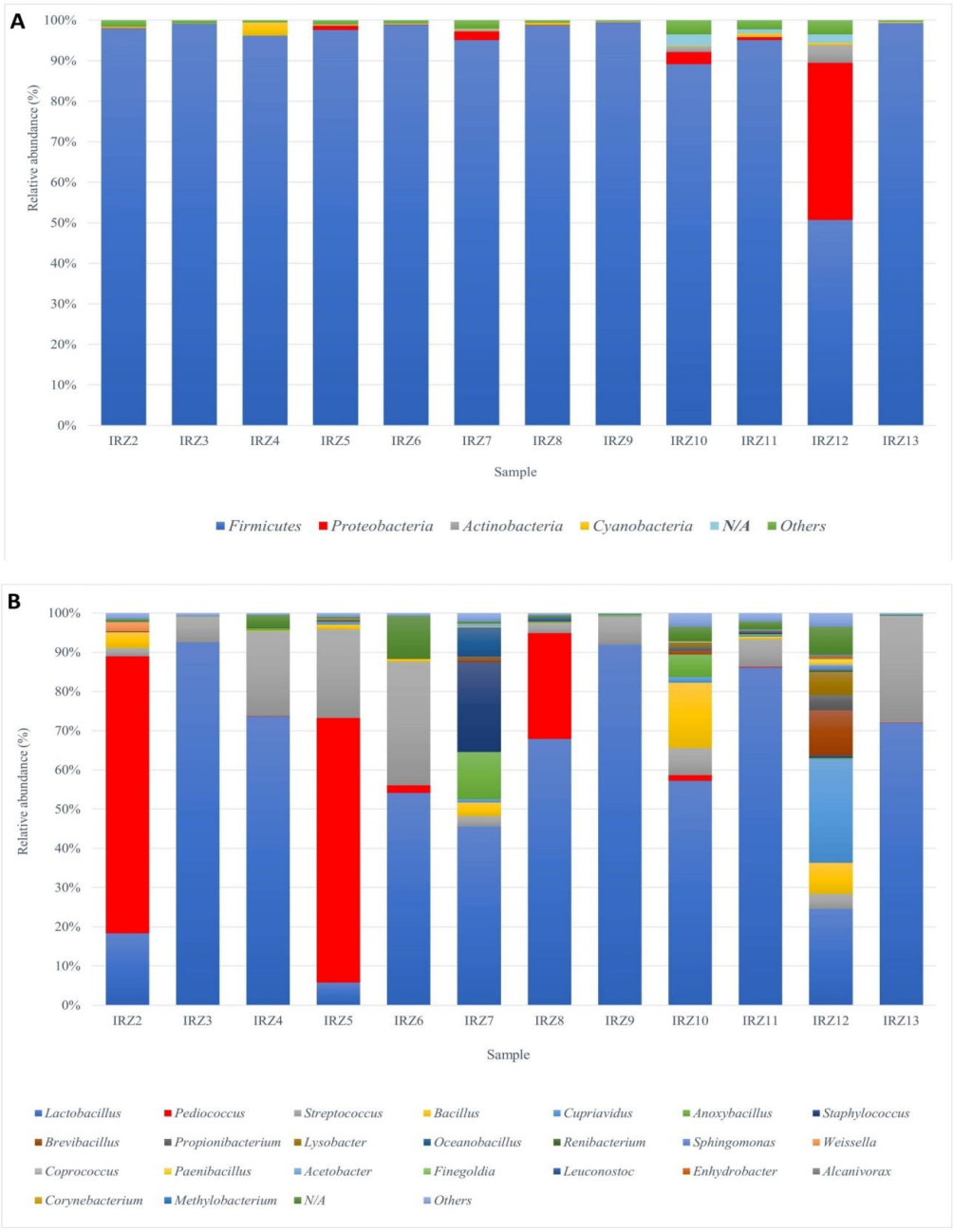

Figure 1.5. Relative abundance of bacterial groups of YC samples at the phylum (**A**) and genus (**B**) level.

A heat map of relative abundance and distribution based on bacterial genera (Figure1.6.) was constructed with the software MultiExperiment Viewer (MeV) (14).A high presence of *Pediococcus acidilactici*, a species frequently found in traditional cereal fermentations but not typical of dairy products(15) ,was observed among the samples and found to be particularly present in three samples (IRZ2, IRZ5, and IRZ8). Interestingly, in these samples, a concomitant

lower abundance of *Lactobacillus* was noticed that could be correlated to some antagonistic activity, possibly linked to the synthesis of antibacterial proteins (bacteriocins), a characteristic quite widespread among pediococci (16) .Indeed, *P. acidilactici* is a well-known bacteriocin (pediocins)-producing species active against Gram-positive bacteria (mainly *Listeria* and some Lactic Acid Bacteria (LAB)) that is of interest in the food industry for its potential application in biopreservation (15, 17).Furthermore, *P. acidilactici* has attracted attention as a promising probiotic, as evidenced by some studies (18, 19) .The genus *Streptococcus* was found consistently in all samples at percentages ranging from 2% (IRZ2) to 31% (IRZ6) of relative abundance. Although 16S rRNA analysis used in this study cannot achieve the species level for this genus, a MegaBLAST search revealed 100% identity with *S. thermophilus* strains, including the type strain ATCC 19258. *S. thermophilus* is naturally present and industrially used worldwide in the manufacture of many homemade and industrial fermented dairy products such as yogurt, *tarag, kurut* and *dahi* (6, 20).

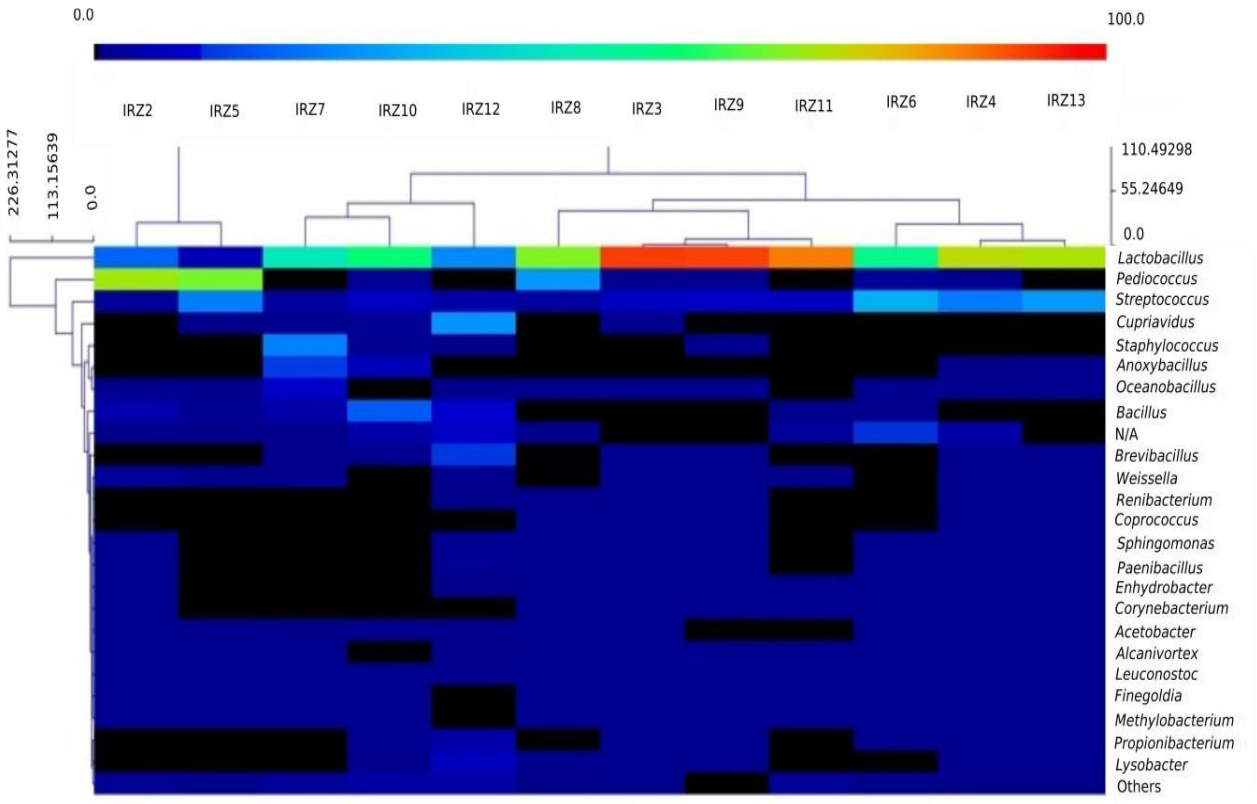

Figure1.6. Heatmap reporting the levels of microbial diversity in terms of genera among YC samples.

1.3.2. Chemical properties of YC samples

The results of chemical analyses, namely pH, protein and lipid contents, and ash and dry matter weights of YC samples are reported in Figure1.7. Regarding pH, all products were acidic due to the fermenting activity carried out by lactic acid bacteria on the milk used to obtain the yogurt that constitutes the basis of this food product. The highest pH (Figure 1.7A) was recorded for sample IRZ4 (5.1) which was the only sample that was significantly different ($p < 0.05$) from all other samples, which gave values below 4.6 (mean: 4.18). Values below pH 4.6 are sufficient to prevent the growth of most pathogenic bacteria(21),thus enhancing the microbiological safety and shelf life of this product.

Regarding dry matter, which represents the weight of a food product from which water and volatile substances have been removed (22, 23),the average percentage considering all YC samples was 91.82%, with values ranging from 88.5% in sample IRZ2 to 93.9% in sample IRZ8 (Figure 1.7B). These very high values are related to the nature of YC, which is substantially a dried product. This makes YC a product that is very different from most milk-based fermented foods which have a much lower dry matter content, such as yogurt (about 13%).

In Figure 1.7C, the percentage composition of the main nutrient categories (namely protein, lipids, ash, and carbohydrates) is reported. While the first three components were determined analytically, carbohydrates were determined by subtraction of these three from the dry matter and it comprises all forms of carbohydrates, including monosaccharides, oligosaccharides, polysaccharides, both soluble and insoluble fiber (i.e., starch and cellulose). As shown in Figure 1.7C, all samples display a similar composition, demonstrating a homogenous manufacturing process adopted among the sampled locations.

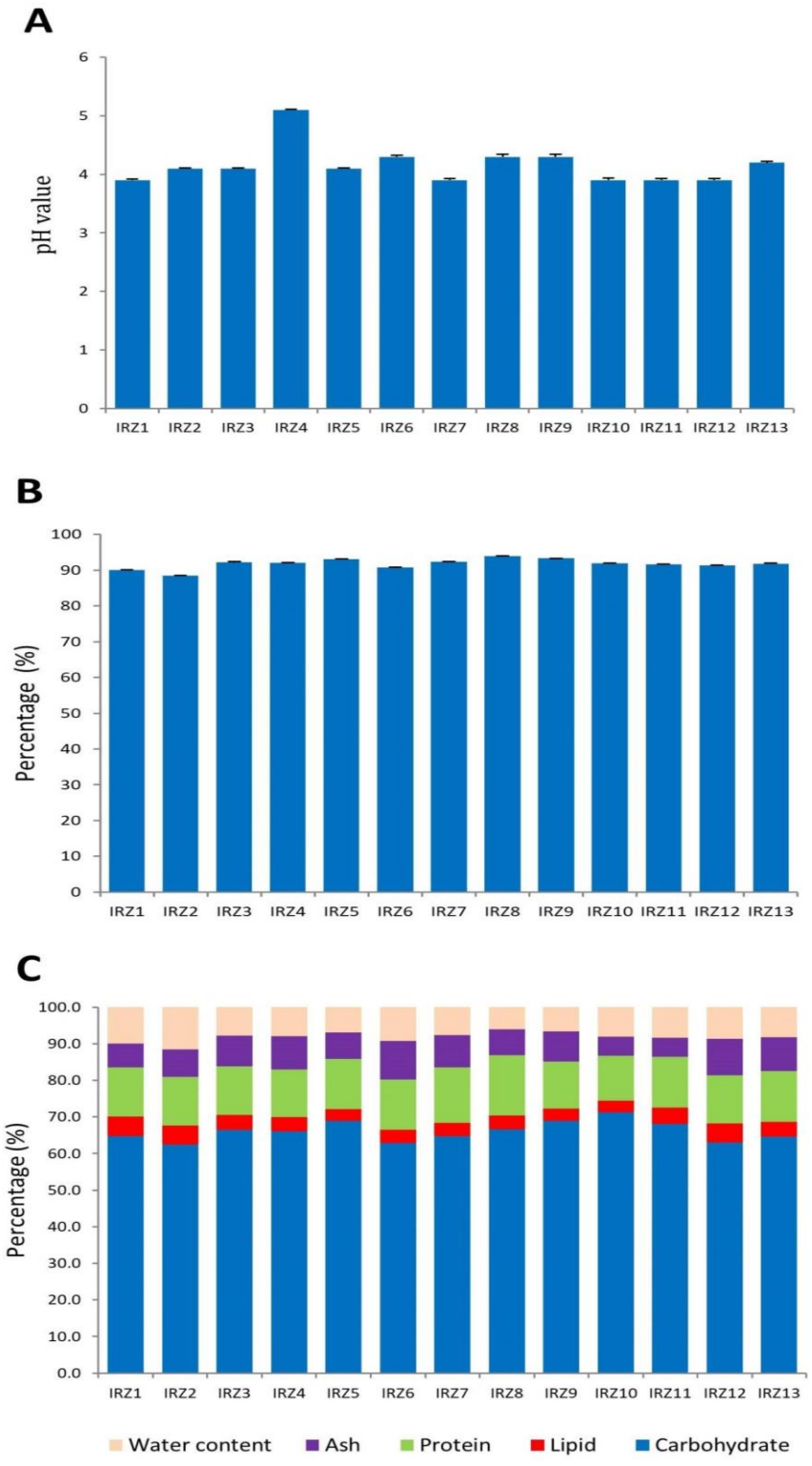

Figure1.7. Chemical properties of YC samples: pH (A); dry matter (B); ash, lipids, protein, and carbohydrates (C).

1.3.3. Mineral content of YC samples

Mean values are 7.91% ± 1.66 for ash, 13.71% ± 1.07 for protein, 4.09% ± 0.73 for lipids and 61% ± 2.13 for carbohydrates. Ash includes the total amount of minerals in the product that play a significant role in nutritional value, quality, and microbiological stability (24). Lipid content is important in the dairy industry since it is a key source of energy and fatty acids. Lipids can be also very beneficial at delivering fat-soluble vitamins, such as vitamin A, D, E, and K (25). Protein plays a key role in the texture and sensory properties of dairy products (25), as well as nutritional and health values (26). Compared to a similar Iranian dried fermented milk product "dried kashk" (27),YC contains three times less protein and about 1.5 times less fat, while it is more abundant in carbohydrates due to the addition of wheat flour during YC production.

YC is consumed as a soup, which is obtained by dissolving about 6 g of powder in 1 L of water. Considering an average serving of about 250 mL (one dish), the contribution of each serving corresponds to about 0.2 g of protein, 0.06 g of lipid, 0.92 g of carbohydrate and 0.12 g of ash (minerals). Compared to yogurt, the most diffuse fermented milk, YC provides about four times more protein, the same amount of lipids, 14 times more carbohydrates and 11 times more ash (i.e., minerals). Indeed, due to the dilution of the powder in the soup, the actual intakes are lower. The mineral content of foods is important in relation to their nutritional value. In the case of YC, this property relies mostly on the characteristics of the milk used to produce the yogurt, which can be related to several factors such as the animal's genetic background, the type of pasture, and the environmental conditions. The mineral content is also dependent on the other ingredients added during the manufacturing of YC, such as the wheat flour, seasonings and salt. We measured the concentration of seven of the most nutritionally relevant minerals, namely calcium (Ca), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), phosphorous (P), and zinc (Zn) ions in all YC samples (Table 1.1). The concentrations of K (mean value: 8038.4 ± 766.1 mg/kg), Mg (mean value: 1655.8 ± 114.0 mg/kg), Mn (mean value: 28.8 ± 3.7 mg/kg), P (mean value: 4059.6 ± 356.4 mg/kg) and Zn (mean value: 27.6 ± 3.3 mg/kg) showed little variation among samples, up to about 10% from the respective means, indicating a quite homogeneous YC composition throughout the regions sampled. Regarding Ca, its variability appears much higher, probably due to the characteristics of the milk used (28) , which represents a major source for this mineral in YC. Na was present in high amounts (mean: 19.16 g/kg) and its variability was high among the samples, from about 10 to 30 g/kg. Although these values are

considerably higher than values reported in the literature regarding other types of “wet” curds (29, 30), another Iranian type of dried curd (27) was also found to contain between 38 and 40 g/kg of NaCl. Due to the lack of a standard recipe, different homemade processes can make use of various amounts of sodium chloride, with the aim of influencing both sensory and safety aspects of YC.

By comparing the average mineral content of yogurt with that of YC, many relevant differences can be detected. Apart from the calcium content (1200 mg/kg in yogurt), which is similar on average to that of YC, all other minerals tested are much more present in YC than they are in yogurt. In detail, K is about five times higher, Mg is 13 times higher, Mn is 720 times higher, and P and Zn are about four times higher. The sodium content of YC is 43 times greater than that of yogurt, but this is not surprising since in yogurt there is no addition of NaCl, differently from YC. Indeed, due to the dilution of the powder in the soup, the actual intakes are lower.

Table 1.1. Mineral content of YC samples (mg/kg dry weight). Results are expressed as means ± standard deviation (SD) (n = 3).

	Ca	K	Mg	Mn	Na	P	Zn
IRZ1	2414.5±78.5	8062.3±129.2	1706±8.3	27.6±0.1	14277.9±150.5	3544.4±27.4	27.4±0.1
IRZ2	1627.1±42	9082.2±219.7	1824.1±49.5	33.6±1	18235.1±569.1	3938.2±147.1	26.7±0.5
IRZ3	2256.9±59.5	7961.4±45.1	1565.8±33.4	22.4±0.2	21761.1±280.6	3838.9±51.5	25.1±0.4
IRZ4	1709.6±79.3	8581.8±265.8	1690±57.5	30.1±1.4	24106.6±1051.1	3789.4±153.8	19.6±0.8
IRZ5	1404.6±40.9	7244.9±73.1	1658±12.3	24.7±0.2	17747.6±187.1	4304.7±38.5	32.7±0.3
IRZ6	1368.4±43.7	7387.5±138.2	1524.7±42.8	27.4±0.4	30069.1±734.7	3520.3±87.8	32.4±0.7
IRZ7	1965.5±31.3	7675.4±169.5	1594.6±45.9	26.3±0.8	22176.2±846.1	4500.7±156.1	28.7±0.7
IRZ8	1359.1±13.6	7923.2±41.6	1835.7±6.9	27.8±0.3	15896.9±67.1	4633.5±34.5	28.7±0.2
IRZ9	2280.1±133.4	8703.7±402.5	1582.6±83.4	25.3±1.4	19659.3±890.3	4204.5±225.7	26.9±1.5
IRZ10	1476.1±73	7379.1±48.6	1666.8±72.2	33.8±0.7	10168.6±69.7	4297.6±47.6	24.3±1.1
IRZ11	1860.1±38.9	7554.7±167	1660.1±58.2	30.9±0.7	8911.9±240.4	4400.6±119.7	28.6±0.8
IRZ12	3035.0±107.7	9689.4±280.6	1770.3±42.6	33.7±0.9	23286.5±376.7	3939.3±92.8	28.2±0.4
IRZ13	1894.7±67.9	7253.5±134.2	1446.4±42.3	31.1±0.9	22817.1±309.2	3862.7±154.6	29.5±0.7
Mean	**1853.1±490.9**	**8038.4±766.1**	**1655.8±114.0**	**28.8±3.7**	**19162.6±5637.7**	**4059.6±356.4**	**27.6±3.3**

1.3.4. Sugar content

We measured the three quantitatively most important sugars in YC, namely lactose, galactose, and glucose. Lactose comes from milk, where it is normally present at a concentration of 3.5–4%, while galactose is excreted from cells of the LAB strains that are unable to utilize it after lactose hydrolysis. The concentration of galactose in YC samples should be taken into consideration with respect to galactose intolerant people. Many people around the world suffer from galactosemia, which is a sort of disorder caused by the deficiency of galactose-1-phosphate uridylyltransferase (GALT) (31).This enzyme is important in the Leloir pathway of galactose metabolism (32).Although morbidity and mortality from galactosemia have been prevented in many countries because of medical screening, in poor districts such as Sistan and Baluchestan provinces, the prevalence could still be high (31) .Glucose comes from plant material, particularly from wheat flour after partial starch hydrolysis. The average concentration of lactose, glucose, and galactose among all samples was 1.28 ± 0.32, 0.98 ± 0.80 and 1.51 ± 0.55 g/100g, respectively (Figure 1.8). Since the average carbohydrate content is quite similar among the YC samples, the differences in carbohydrate content recorded are likely to be due to microbial activity during fermentation.

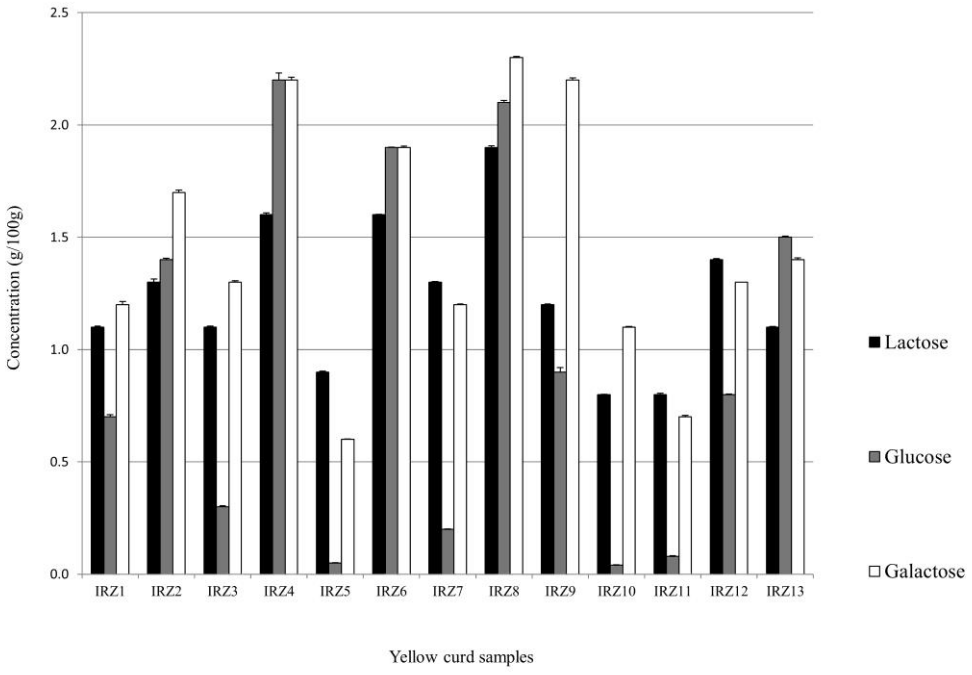

Figure1.8. Concentration of sugars in YC samples determined by HPLC analysis. Results are expressed as means ± SD (n = 3).

Considering the three most abundant LAB genera found in YC samples, all streptococci of dairy origin are able to utilize lactose, whereas only some species of *Lactobacillus* can degrade it, and lactose is normally not metabolized by pediococci (33, 34). Among the lactose-utilizing strains, the capability to use galactose is strain dependent (35). Glucose is metabolized by pediococci and by lactobacilli, while streptococci use it slowly or not at all, depending on the strain (35, 36). By comparing carbohydrate values with microbiome analysis, we were not able to find any significant correlation with a bacterial category. Therefore, the differences in the carbohydrate pattern are most likely due to the presence of different strains with different metabolic capabilities regarding sugar metabolism. Such differences appear to be quite relevant, particularly in the case of glucose.

1.4. Conclusion

This work represents the first study on yellow curd, a widespread fermented food largely consumed in some regions of Iran. By using both a canonical and a molecular approach we were able to characterize the microbiota of YC. In particular, we detected that most bacteria were not alive in the final product, stored as powder, with the exception of a considerable number of putative *Bacillus* spore. In addition, by 16s RNA sequencing, we were able to determine the bacterial categories that developed during the fermentation process. These bacteria, although no longer alive, contributed to define the characteristics of the products, its safety and possibly to enrich it by producing valuable compounds. This latter possibility was none investigated and could be valuable matter for further studies. We also measured the main nutritional components and found that the powder has a good content of protein, lipid and it is rich in carbohydrate, mainly probably starch and cellulose from plant material and from wheat flour. Unfortunately, the dilution of the powder used to prepare the soup, which represents the common way of consumption, considerably reduced the nutritional contribution. In this sense, an interesting future development could be the use of the YC powder as base for the preparation of novel functional foods.

Chapter 2

Genomic and phenotypic evaluation of potential probiotic *Pediococcus* strains with hypocholesterolemic effect isolated from traditional fermented food

Abstract

The use of probiotic microorganisms in food with the aim to confer health benefits to the host is one of the most critical roles of functional foods. Many pediococci bacteria frequently related to the meat environment, have technological properties and are therefore commercially used as starter in the production of fermented meat products, such as different types of sausages. In this study, different lactic acid bacteria were isolated, identified to the species level, and then evaluated for their safety and functionality as possible probiotics. Different properties, such as resistance to simulated human gastrointestinal conditions, antimicrobial activity and cholesterol-lowering effects, have been studied. Finally, the complete genome of one strain, namely *P. acidilactici*IRZ12B, which showed interesting features as a promising probiotic candidate, was sequenced and further studied. The results revealed that IRZ12B possesses interesting probiotic properties, particularly cholesterol-lowering capability and antimicrobial activity. *In-silico* analysis evidenced the absence of plasmids, transmissible antibiotic resistance genes, and virulence factors. We also detected a bacteriocin coding gene and a cholesterol assimilation-related protein. The phenotypical and genomic outcomes described in this study make *P. acidilactici* IRZ12B a very interesting cholesterol-lowering potential probiotic strain to be considered for the development of novel non-dairy-based functional foods.

2.1. Introduction

The term 'probiotic' was derived from a Greek word that means "for life". According to the Food and Agriculture Organization (FAO) and the World Health Organization (WHO), probiotics are defined as "live microorganisms which, when ingested in adequate amounts, confer a health benefit on the host"(37). Presently, the use of probiotics is being increasingly adopted in the production of functional foods. The probiotic bacteria mostly used in functional products belong to the genera *Lactobacillus*, *Bifidobacterium*, *Pediococcus*, *Enterococcus*, *Bacillus*, *Streptococcus* and *Escherichia* (38–40). In order to evaluate the probiotic potential of a strain, experts from FAO, WHO, and European Food Safety Authority (EFSA) have established specific guidelines (41). According to these recommendations, besides conferring some health benefits to humans, a newly proposed strain must be able to withstand passage through the human gastrointestinal tract, being able to tolerate gastric conditions and grow in the presence of bile (42). Over the last twenty years, probiotic

bacteria have gained importance due to the continuously increasing scientific evidence of their beneficial effects on human health. Among other actions, probiotic strains can help reducing the risk of heart diseases caused by high levels of cholesterol in the blood (43). They can decrease the level of cholesterol in different ways, such as binding the cholesterol molecule to their cell surface and then incorporating it into the cellular membrane (44),by deconjugation of bile *via* bile salt hydrolysis activity or by cholesterol conversion to coprostanol (45).The antimicrobial activity of probiotics against pathogens is another interesting feature, frequently found inside this group of bacteria. Bacteriocin-producing probiotics are widespread among lactic acid bacteria (LAB)(46). Pediocins are a group of class II-a bacteriocins produced by *Pediococcus* strains, endowed with antimicrobial activity against some Gram-positive bacteria, including *Listeria* (47). Pediococci are frequently isolated from the meat environment and several strains have shown interesting technological properties in production of fermented meat products and are used as starters. If they possess probiotic features, they could become interesting for the production of novel non-dairy-based functional food.

Officinal plants as natural medicines have a long history from ancient people, and the usage of medicinal plants as cure or treatment has been handed down from one generation to the other from all over the world (48). *Echinacea* from the family Asteraceae is well known as a traditional medicinal plant, and it has many benefits. Firstly people from North America were using it for diseases, infections, sores, and injuries. Recent studies showed that *Echinacea* also has other benefits such as antimicrobial and antiviral activity, anti-inflammatory, boosting the immune system, antioxidant activity (49).

Malva, from the family Malvaceae, is another famous traditional plant that mostly comes from Asia, Europe, and North Africa. Recent studies indicate that it also benefits human health through anti-inflammatory, antioxidant, anti-complementary, anti-cancer, and skin tissue integrity activity (50).

Calendula from Asteraceae family, is another famous medicinal plant from southwestern Asia, Western Europe, Macaronesia, and the Mediterranean, mostly known as marigolds. *Calendula* has shown fascinating broad therapeutic effects such as anti-inflammatory, anti-diabetes, and treating skin diseases, wounds, and herpes (51). During the last decade, development and innovation in food biotechnology carried to an estimated growth tendency rate of 28% per year of novel products with functional claims (52)**.** Nowadays, food plants, are considered a simple nutritional intake and a potential source of healthy and natural products due to different natural molecules and active compounds (53). The research towards

non-dairy functional foods is expanding due to the evolving trend of vegetarianism, the increasing prevalence of lactose intolerance, the effects of dairy products on cholesterol levels, and the demand for foods that improve well-being and reduce the risk of disease (52). In particular, a significant interest in natural antimicrobial, antioxidant, anti-inflammatory, and immuno-modulatory compounds encourages the nutraceutical industry to exploit plant potential even more.

In this study, we have isolated and identified some potential probiotic strains from a traditional Iranian fermented food and have studied them for the presence of potential probiotic properties, in particular the capability to lower cholesterol levels. Finally, the best strain has been used to produce herbal functional fermented foods.

2.2. Material and Methods

2.2.1. Sample collection and isolation of bacteria

A total of 14 yellow curd samples were collected from different local households' traditional markets of the Sistan and Baluchestan province, in South East of Iran (54). Isolation of bacteria from the samples was carried out using 5 g of powder from each sample inoculated into 50mLof MRS (De Man Rogosa Sharp, Sigma-Aldrich, Saint Louis, MO, USA) broth and incubated anaerobically without agitation at 37°C for 48h. Following incubation, serial dilutions were plated on MRS agar and incubated anaerobically at 37°C for 48h to produce separated colonies. Only 6 out of 14 samples produced colonies on plates. One representative colony was taken from samples IRZ6 and IRZ7, that produced colonies with the same morphology, while 2 colonies each were collected from samples IRZ8, IRZ12, IRZ13, and IRZ14 that showed two different morphologies on plates .All isolates were subjected to Gram staining, microscope inspection for cell morphology determination, catalase and oxidase tests. Stock cultures were prepared in MRS broth containing 25% (v/v) glycerol and kept at -80 °C until further use.

2.2.2. DNA extraction and RAPD-PCR analysis

DNA extraction was done according to Tarrah *et al*.(55)with slight modifications. Briefly, 5mL of overnight bacterial cultures were centrifuged at 5500 g for 5 min, washed 2 times with5 mL sterile PBS (PBS; NaCl 8.0 g/L, KCl 0.2 g/L, Na_2HPO_4 1.44 g/L, KH_2PO_4 0.24

g/L, pH 7.4, Sigma-Aldrich) and added to50µL of lysis buffer (NaOH 0.05 M + SDS 0.25%). The suspension was vortexed for 2 min and then incubated at 94°C for 15 min using a thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). Finally, the supernatant, obtained after centrifugation at 13,000g for 10 min, was collected and diluted1:100 in ultrapure sterile water. The RAPD-PCR analysis was performed using primer M13 (5' GAGGGTGGCGGTTCT 3') according to the protocol by Andri ghetto *et al.* (56). Electrophoretic profiles were analyzed using the software package, Gel Compar Version 4.1 (Applied Maths, Kortrijk, Belgium), and a dendrogram was drawn by using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) based on the Pearson product-moment correlation coefficient.

2.2.3. Amplification and sequencing of 16S rRNA

Strains identification was carried out by16S rRNA sequencing using the universal primers 27F (5′ GAGTTTGATCNTGGCTCAG3′) and 519R(5′GWNTTACNGCGGCKGCTG3′)(57, 58)(BMR, Padova, Italy).Amplification and sequencing of the amplified products were done as previously described by Clarridge [16], and 16S rRNA sequences were compared with those available in the GenBank database using the BLASTN program (59) at the National Center for Biotechnology Information server.

2.2.4. Determination of antibiotics minimum inhibitory concentration

Determination of Minimum Inhibitory Concentration (MIC) of eight antibiotics among those recommended by the European Food Safety Authority(EFSA, 2008)[18]was carried out according to Wiegand et al. (61) using the microdilution method in 96-well microtiter plates. Chloramphenicol, streptomycin, ampicillin, kanamycin, tetracycline, erythromycin, vancomycin and gentamycin (Sigma-Aldrich) were dissolved in ISO-Sensitest broth (Sigma-Aldrich) plus 10% MRS (Sigma-Aldrich) and distributed as 2-fold serial dilutions in the microtiter plate wells. Each microtiter well was then inoculated with the bacterial culture to a concentration of 5×10^5 CFU/mL. The MIC was determined as the antibiotic concentration in the first well with no visible growth after 20 h of incubation. The test was performed in triplicate.

2.2.5. Hemolytic activity test

Twenty µL of fresh bacterial cultures were spotted onto MRS plates containing 5% (w/v) sheep blood (Thermo Fisher Scientific, United States) and incubated at 37°C for48 h. Hemolytic activity was revealed by the presence of a clear halo surrounding the colonies. The experiment was performed with three biological replicates, each one in triplicate.

2.2.6. Resistance to simulated gastrointestinal conditions

Strains susceptibility to simulated gastrointestinal conditions was determined as previously described by Tarrah et al. (62). Cell suspension obtained after two subcultures to activate the cells completely were subjected to simulated gastric conditions for 1 h. Susceptibility to intestinal conditions was evaluated right after the gastric passage for a further 3 and 5 h. Microbial viability after each step was checked out using the micro drop technique by placing 20 µL of each dilution on the surface of a MRS agar plate. The experiment was repeated three times with three technical replicates each.

2.2.7. Measurement of cholesterol assimilation

Cholesterol assimilation by bacterial cells was determined using the colorimetric method described by Miremadi et al.(63) With a slight modification. Initially, 100µL of an overnight culture of bacterial strains were grown in 10 mL MRS broth containing 0.3% bile-salt ox gall (Sigma-Aldrich) and 100µg/mL of filter-sterilized cholesterol (Sigma-Aldrich) and incubated at 37 °C for 24 h. After incubation, cultures were centrifuged at 5,000 g for 15 min at 4°C, and 1mL of the supernatant was collected to calculate bacteria cholesterol assimilation. One milliliter of the supernatant was added to 1 mL of 33% (w/v) KOH and 2 mL of 96% ethanol, vortexed for 1 min and incubated at 37 °C for 15 min. The solution was then equilibrated to room temperature prior to adding 2 mL of sterile water and 3 mL of hexane. The mixture was vortexed for 1 min and left to stand for phase separation. The upper hexane layer (approximately 2mL) was transferred into a 24 well microtiter plate and evaporated under a nitrogen gas atmosphere. Then, 2 mL o-phthalaldehyde (0.5 mg/mL in glacial acetic acid, Sigma-Aldrich) were added to the wells and mixed gently to dissolve the residues completely. To this, 0.5 mL of 98% sulphuric acid (Sigma-Aldrich) were added, vortexed for 1min, and incubated for 10 min at room temperature before measuring the absorbance at 550nm with a microtiter plate reader (Spark 10M, Tecan GmbH, Grödig, Austria). Cholesterol concentration was determined using a standard curve from 0 to 100µg/mL. The

experiment was repeated 2 times, with 3 technical replicates. The capability to assimilate cholesterol was reported as the percentage of cholesterol removed after 24 h.

2.2.8. Antimicrobial activity determination

Twenty µL of overnight cultures were spotted on the surface of two different MRS plates and incubated overnight. In one of the two plates, 2 µL of proteinase K solution (20 mg/mL, pH 8) were spotted close to the cells spot to detect the production of bacteriocin compounds and incubated for 1 h. Successively, a lawn of 4 mL of BHI soft agar (Sigma-Aldrich) containing 400µL of an overnight culture of the indicator strains, namely *Escherichia coli* DH1, *Pseudomonas fluorescens* DSM50090, *Staphylococcus xylosus* DSM20266, *Bacillus subtilis* DSM10, *Bacillus amyloliquefaciens* DSM7 and *Listeria innocua* DSM 20649 were poured on the top of the existing solid medium and plates were then incubated at 37 °C for 24 h. After incubation, plates were inspected for the presence of zones of growth inhibition surrounding the colonies. The presence of inhibition haloes in the MRS plate and the absence of haloes for the same strain in the protease-containing plate indicates the proteinaceous origin of the antimicrobial compound (64).

2.2.9. Genome sequencing, assembly, and annotation

Genomic DNA from *P. acidilactici* strain IRZ12B was extracted using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) according to the manufacturer instructions. The quality assessment of the extracted DNA was evaluated spectrophotometrically (Spark 10M). Genome sequencing was carried out using the paired-end sequencing technology with an Illumina MiSeq sequencer (BMR).PATRIC database server 3.6.4 was used to assemble the raw reads using the Unicycler assembler set up on default parameters (65). The Rapid Annotation using Subsystems Technology (RAST) was used to predict and annotate the assembled genome (66).

2.2.10. Bioinformatical analysis and comparative genomics

A graphical genome map of *P. acidilactici* IRZ12B was constructed using the PATRIC 3.6.4 server (65)after scaffolding the assembled contigs using medusa webserver (67) and the type *strain P. acidilactici* DSM 20284^T as the reference genome.

Genomes of 28 different strains from 10 *Pediococcus* species, including the type strain of each species available in Genbank (NCBI) along with *P. acidilactici* IRZ12B,were used to

construct the phylogenetic tree (Table 2.1). A fragmented all-against-all comparison using "BLASTN" mode was carried out with Gegenees software (68), setting the parameters on 500/500 (frag-size/slide-size) to compute the distance among the strains. Then, splitTree5 software was used to cluster the strains and create the phylogenetic tree using the neighbor-joining (NJ) method (69).

Comparative genomic analysis using the whole proteins content of the *P. acidilactici* cluster, including strains DSM 20284^T, SRCN103444, GS1, E24, PMC48, and IRZ12B was done using the web platform Orthovenn (70) with default parameters. Strain genomes annotation was performed using the Rapid Annotation using Subsystems Technology (RAST), and the whole protein content from the genome of each strain was used for Pan/core-genome analysis.

Plasmid Finder 2.0 and Res Finder 3.2 servers were used to assess the presence of plasmids and transmissible antibiotic resistance genes on the genome, respectively (71, 72). Genes related to bacteriocin production in *P. acidilactici* IRZ12Bwere also identified using the BAGEL4 server (73).

Table 2.1. *Pediococcus* genomes used for genetic analyses.

Strains	Accession number
P. acidilactici GS1	NZ_QNGC00000000
P. acidilactici SRCN103444	NZ_CP035266
P. acidilactici DSM20284T	NZ_AEEG01000000
P. acidilactici E24	NZ_SDQW00000000
P. acidilactici PMC48	NZ_CP050079
P. acidilactici IRZ12B	JABXOH000000000
P. argentinicus DSM23026T	NZ_JQCQ00000000
P. cellicola DSM17757T	NZ_JQBR00000000
P. claussenii TMW2.54	NZ_CP014936
P. claussenii TMW2.53	NZ_CP014933
P. claussenii DSM14800T	NZ_JQBB00000000
P. damnosus MBPD14610	NZ_LTEA00000000
P. damnosus DSM20331T	NZ_JQBD00000000
P. damnosus LMG28219	NZ_JANK00000000
P. damnosus VTTE123212	NZ_NAER00000000
P. damnosus VTTE123216	NZ_NAEQ00000000
P. ethanolidurans DSM22301T	NZ_JQBY00000000
P. ethanolidurans CGMCC1.3889	NZ_FOGK00000000
P. inopinatus DSM20285T	NZ_CP019981
P. inopinatus WIKIM15	NZ_BBIM00000000
P. parvulus 2.6	NZ_LXND00000000
P. parvulus NBRC100673	NZ_BJWE00000000
P. pentosaceus SL001	NZ_CP039378
P. pentosaceus JQI7	NZ_CP023655
P. pentosaceus DSM20336T	NZ_JQBF00000000
P. pentosaceus SL4	NC_022780
P. pentosaceus FBL2	NZ_LSFE00000000
P. stilesii DSM18001T	NZ_JQBX00000000
P. stilesii FAM18815	NZ_VBTH00000000

2.2.11. Plant selection screening

Two hundred milliliters medium were prepared by addition of 10g ground medicinal plants (*Malva, Calendula, Echinacea,* Olivo, Vite, Santoreggia) in 190 mL Milli-Q water (5%), autoclaved at 121°C for 20 min, and the supernatant was collected by centrifugation at 5500 for 10 min. Two milliliters of an overnight culture of *P.acidilactici* IRZ12B strain were used to inoculate each abovementioned media (1% culture), which was immediately transferred to a 96-well plate (200 µL/well) and incubated at 37°C for 24h. The optical density was recorded every hour using Tecan Spark.

2.2.12. *Malva*, *Calendula*, *Echinacea* fermentation

The main fermentation was done by using a 1% overnight culture of *P. acidilactici* IRZ12B, inoculated in *Malva, Calendula,* and *Echinacea* media, and incubated at 37°C for 24h. One medium from each herb without bacterial inoculation was included in all experiments as non-fermented control. After the fermentation step, the corresponding bacterial load was checked and recorded using the 10-fold Serial dilutions technique, followed by plating on MRS agar medium, and incubation at 37°C for 24h. The initial and final pH for each medium was recorded as well.

2.2.13. Different concentration preparation of lyophilized supernatants

Eighty milliliters of fermented and non-fermented herbal supernatant were collected by centrifugation at 5500g for 10 min. The pH was adjusted to 7.0 using 1N NaOH, filtered (0.22µm), and lyophilized using a freeze drier machine (Edwards Modulyo Freeze Dryer)**.** Later, 0.1 g of each lyophilized powder was added to 10 mL of Dulbecco's Modified Eagle Medium (DMEM) and filtered for the second time. Finally, different concentrations (25, 50, 100, 200, and 500 µg/mL) for differentiated Caco2 cells and 250µg/mL, 500µg/mL, and 1000µg/mL for MCF-7 cells were prepared for further analysis.

2.2.14. Cytotoxicity effect on intestinal cell lines

To evaluate the cytotoxic effect of fermented plants on normal human cells, Caco-2BBe1 cells (healthy intestinal cells) were seeded into six-well plates at a concentration of 10^5 cells/well, and grown for 15 successive days after reaching confluency. Fully differentiated Caco-2BBe1 cells were treated with different concentrations (25, 50, 100, 200, and 500 µg/mL) of non-fermented/ fermented plants (diluted in DMEM) and incubated at 37°C for 48 h under a 5% CO_2 atmosphere (74). After treatment, cells were collected using Trypsin-EDTA 0.25% (Sigma-Aldrich) in falcon tubes, centrifuged, and the supernatant was discarded. Ultimately, cells were re-suspended in a fresh DMEM medium, stained with Trepan blue dye (0.4%, Gibson), and counted under the microscope to determine the viability percentage. This experiment was done in 3 biological replicates containing 2 technical replicates.

2.2.15. Cytotoxicity effect on Human Breast cancer cells (MCF -7)

To evaluate the cytotoxic effect of fermented plants on human breast cancer cells, MCF-7 cells were seeded into six-well plates at a concentration of 10^5 cells/well using DMEM medium together with 10% FBS and 1% penicillin/streptomycin. Plates were incubated at 37°C incubator containing 5% CO2 for 72h.

MCF-7 cells were treated with different concentrations (250µg/mL, 500µg/mL, and 1000µg/mL) of non-fermented/ fermented plants (diluted in DMEM) and incubated for 48h in the same condition. The control wells were also treated with 1 mL of fresh DMEM medium. After the treatment, the cells were stained as already explained above and counted by a TC20 automated cell counter (Bio-Rad, Hercules, CA, USA). This experiment was done in 3 biological replicates containing 2 technical replicates.

2.2.16. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using GraphPad Prism software (version 7, GraphPad Software, Inc., San Diego, CA).

2.3. Results and Discussion

2.3.1. Strains isolation and identification

Kashk Zard is a traditional Persian fermented food, prepared with a combination of homemade yogurt, wheat flour, different local spices (dill, coriander, cumin, turmeric, garlic), aromatic herbs, and salt. The mixture is then let to air dry completely and then ground into a yellow powder that is used to prepare soups [12]. Since this procedure determined the inactivation of most microorganisms, we were able to rescue only a very limited number of viable bacteria, evidently able to withstand very stressful conditions, that makes it particularly interesting to better characterize them. Thirteen bacterial colonies were isolated from MRS agar plates on the basis of different colony morphology. All isolates resulted Gram-positive, catalase- and oxidase-negative. Except for IRZ14B, which was rod-shaped, all isolates were cocci. A RAPD-PCR analysis was performed to discriminate different strains on the basis of their fingerprints (Figure2.1).

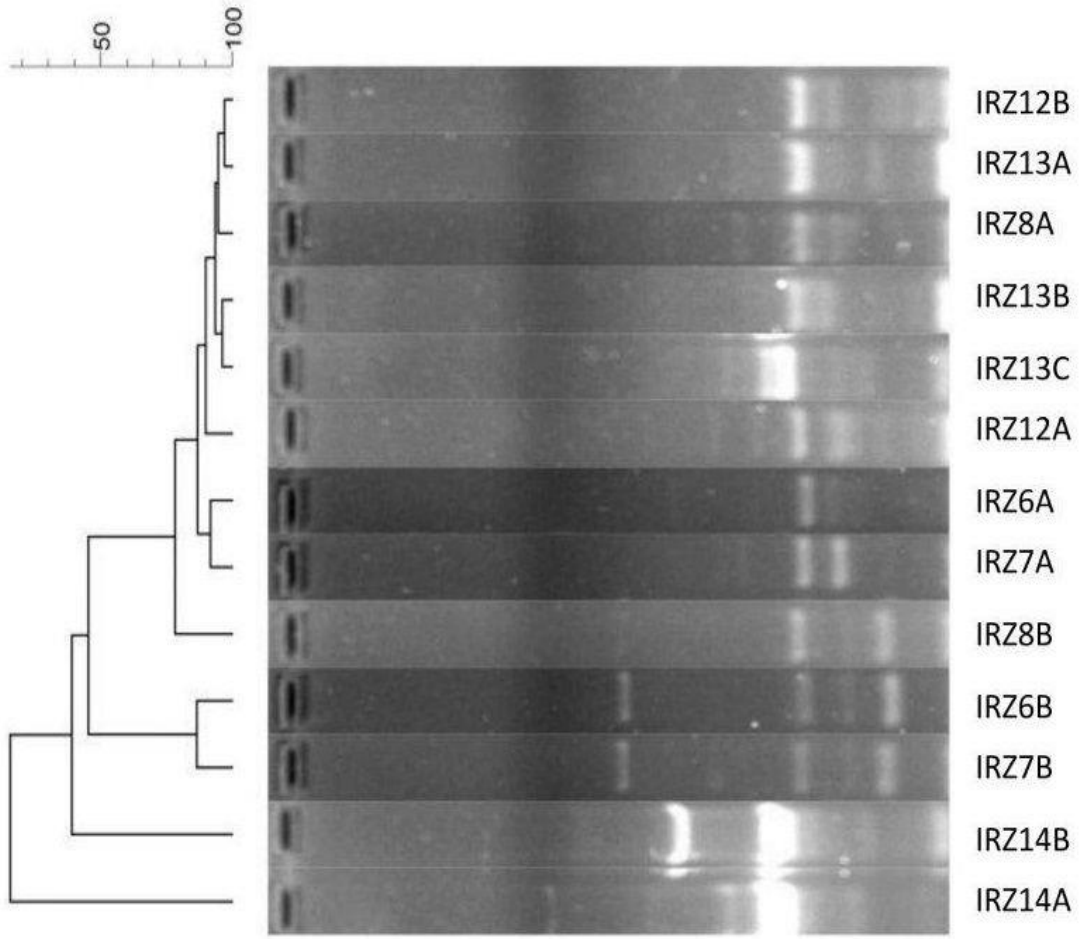

Figure 2.1. Cluster analysis of RAPD-PCR fingerprints of isolates from *Kashk Zard.*

According to the UPGMA analysis, isolates IRZ6B, IRZ7A, IRZ13B, showing profiles similar to IRZ7B, IRZ6A and IRZ13A, respectively, were eliminated. The identification of the remaining 10 strains by partial 16S rRNA sequence analysis, detected seven *Pediococcus acidilactici*, two enterococci (*Enterococcus hirae* and *E. faecium*) and one *Lactobacillus pontis* strains (Table 2.2)

Table 2.2. Strains identification, as determined by partial sequencing of 16S rRNA.

Strain ID	DNA Identity (%)	Species	Accession number
IRZ6A	99.08	*Pediococcus acidilactici*	MN189972
IRZ7B	98.55	*Pediococcus acidilactici*	MN189973
IRZ8A	98.71	*Pediococcus acidilactici*	MN189974
IRZ8B	98.90	*Pediococcus acidilactici*	MN189975
IRZ12A	98.51	*Pediococcus acidilactici*	MN189976
IRZ12B	97.83	*Pediococcus acidilactici*	MN189977
IRZ13A	98.74	*Pediococcus acidilactici*	MN189978
IRZ13C	99.25	*Enterococcus hirae*	MN189979
IRZ14A	99.62	*Enterococcus faecium*	MN189980
IRZ14B	99.44	*Limosilactobacillus pontis*	MN189981

All the isolated strains belong to species included in the lactic acid bacteria (LAB) group and most (7 out of 10) to the species *Pediococcus acidilactici*, which is a species with the Qualitative Presumption of Safety (QPS) status, according to EFSA(75), thus making our strains potentially suitable for their use in foods.

2.3.2. Determination of the minimum inhibitory concentration (MIC)

MIC determination for eight antibiotics of human and veterinary importance was carried out for all strains (Table 2.3). Two main categories of antibiotics are generally recommended by EFSA(76) for the presence of possible resistance, namely inhibitors of cell-wall synthesis (ampicillin and vancomycin) and of protein synthesis (chloramphenicol, gentamicin, streptomycin, kanamycin, tetracycline, and erythromycin). All strains were susceptible to chloramphenicol, gentamicin, streptomycin, kanamycin, tetracycline, erythromycin, and ampicillin, while resistance to the highest concentration of vancomycin, i.e.,64 μg/mL, was recorded for all strains.

In addition to the QPS status, a safety assessment must be done at strain level and, among these, ß-hemolytic activity and antibiotic resistance raise the major safety concerns (77, 78). In particular, determination of the antibiotic resistance pattern is crucial for a potential starter or probiotic bacteria to avoid the possibility of horizontal gene transfer to other bacteria, particularly to pathogens (35). Although the lack of acquired resistance against therapeutically used antibiotics is mandatory, the presence of natural (non-transmissible) antibiotic resistance

could be, on the contrary ,considered beneficial for conferring tolerance towards damage induced by antibiotic treatments (79).The MIC tests revealed that all *Pediococcus*, *Enterococcus* and *Lactobacillus* strains were sensitive to the antibiotics tested, according to the breakpoint values indicated by EFSA, with the sole exception of vancomycin. Vancomycin resistance represents a serious issue for lactic acid bacteria, particularly for enterococci (80). However, the resistance to vancomycin is also frequently found to be intrinsic (natural resistance, non -transmissible), thanks to the low permeability of the cell wall to the large aminoglycoside molecules(81–84). In addition, our *in-silico* analysis on IRZ12B genome did not reveal the presence of genes related to vancomycin resistance.

Table2.3. MIC values for human and veterinary relevant antibiotics. Breakpoints values (μg/mL) suggested by EFSA for each drug are reported in parentheses. Strains with MIC higher than the breakpoint (in bold) are considered resistant.

Antibiotics	IRZ6A	IRZ7B	IRZ8A	IRZ8B	IRZ12A	IRZ12B	IRZ13A	IRZ13C	IRZ14A	IRZ14B
Vancomycin	**>64(4)**	**>64(4)**	**>64(4)**	**>64(4)**	**>64(4)**	**>64(4)**	**>64(4)**	**>64(4)**	**>64(4)**	**>64(4)**
Kanamycin	64(64)	64(64)	64(64)	64(64)	64(64)	64(64)	64(64)	64(64)	64(64)	32(128)
Ampicillin	2(4)	2(4)	2(4)	2(4)	2(4)	2(4)	2(4)	2(2)	2(2)	2(4)
Tetracycline	4(8)	4(8)	4(8)	4(8)	2(8)	2(8)	4(8)	4(4)	2(4)	2(8)
Erythromycin	<0.125(1)	<0.125(1)	0.5(1)	<0.125(1)	<0.125(1)	<0.125(1)	<0.125(1)	1(4)	1(4)	0.5(1)
Chloramphenicol	4(4)	4(4)	4(4)	4(4)	4(4)	4(4)	4(4)	4(16)	4(16)	4(4)
Streptomycin	32(64)	32(64)	32(64)	32(64)	32(64)	32(64)	32(64)	32(128)	32(128)	32(64)
Gentamycin	4(16)	4(16)	8(16)	4(16)	4(16)	4(16)	4(16)	8(32)	8(32)	8(16)

2.3.3. Hemolytic activity test

The assessment of blood hemolytic activity conducted on MRS plates containing sheep blood indicated that none of the strains possess the β-hemolytic activity, and all strains were therefore considered as γ-hemolytic (i.e., without hemolytic activity).

2.3.4. Resistance to simulated gastrointestinal conditions

All strains exhibited a very good resistance level to the incubation in simulated gastric juice by showing a loss of viability lower than 0.2 log (Figure2.2). Contrary, regarding intestinal conditions, two kinds of behavior were recorded. Strains IRZ6A, IRZ8A, IRZ12A, IRZ12B, and IRZ14B evidenced a low viability loss (<1 log) after prolonged (300 min) exposure. This value was more than 2.5 log better than that of the commercial strain *L. rhamnosus* GG, used as reference. In addition, *L. rhamnosus* GG was the only strain showing a markedly different behavior between 3 and 5 h of incubation in gastrointestinal juice. On the other side, strains IRZ7B, IRZ8B, IRZ13A, IRZ13C and IRZ14A showed a weak resistance to gastrointestinal conditions, since just after 180 min they had approximately3 logs decrease in viability .Based on these results, the best strains, i.e. IRZ6A, IRZ8A, IRZ12A, IRZ12B and IRZ14B, were selected for successive analyses. After the safety assessment, the capability to resist gastrointestinal conditions is fundamental for a putative probiotic strain, according to the definition of "probiotic" as "able to reach the gut and produce beneficial effects on the host" (85). The human stomach pH during fasting can vary from 1.3 to 2.5 and can increase up to 4.5 soon after a meal (86). It is well known that tolerance to gastrointestinal transit is strain-dependent, thus giving a good incentive to seek more robust strains in this respect for the foods and healthcare industry(87). In our study, *Pediococcus* and *Lactobacillus* strains showed a strong resistance to simulated gastrointestinal conditions. This outcome is in agreement with previous studies that reported high survival rates for *Pediococcus* and *Lactobacillus* probiotic strains (88–90) . The capability to withstand strong acidic conditions is increased in fermenting bacteria by the activity of the F_0F_1-ATPase (91). This complex enzymatic functions as a membranous channel for proton translocation to create membrane potential(92), which can help in reducing cytoplasmic proton concentration .In addition, probiotic bacteria can also deconjugate bile-salt secreted by the liver thus becoming more resistant to the gut conditions(93).

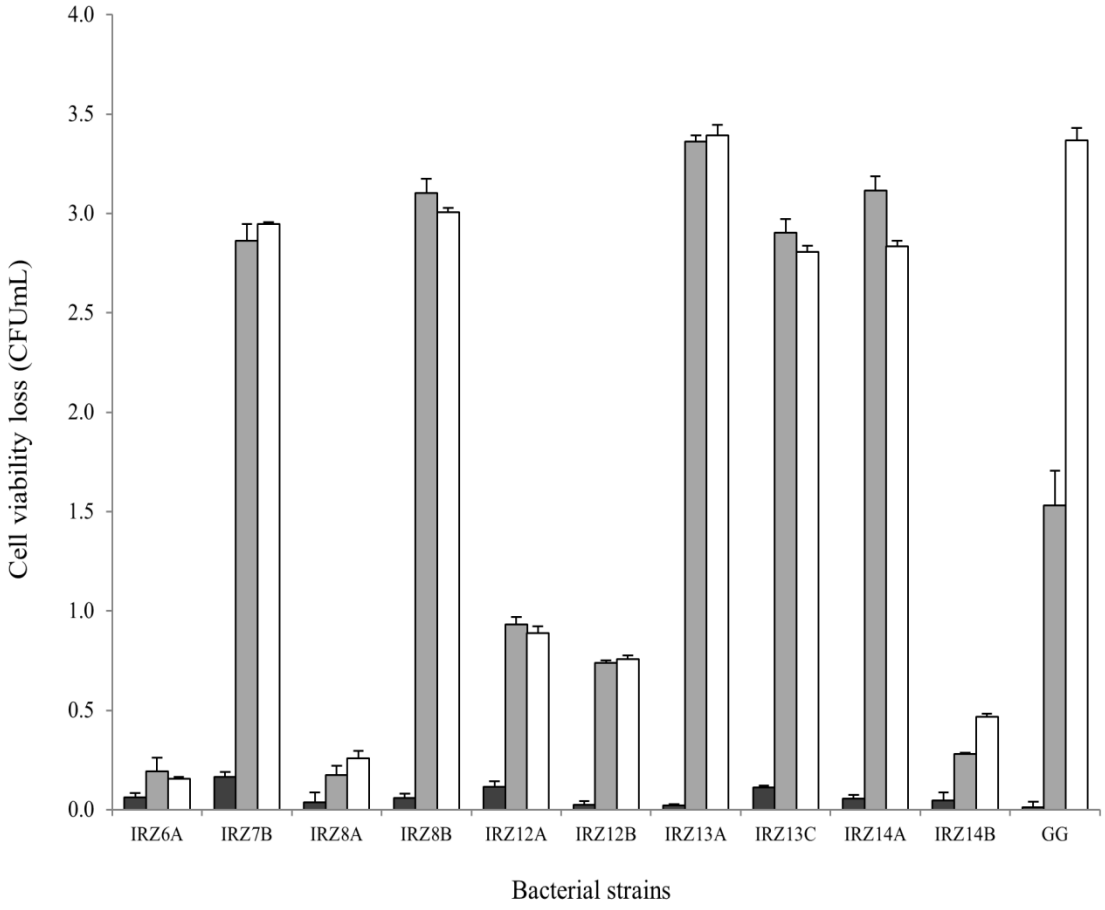

Figure 2.2. Cell viability loss of LAB strains upon exposure to simulated gastrointestinal conditions. Black bars: after 1 h incubation (gastric test); grey bars: after 3 h incubation (short gastrointestinal test); white bars: after 5 h incubation (prolonged gastrointestinal test). Results are expressed as means ± SD (n=3) of viable cells.

2.3.5. Measurement of cholesterol assimilation

It has been reported that the activity of probiotics in the human gut can be part of therapeutic strategies to contrast metabolic disorders, such as hypercholesterolemia and obesity (94). Some probiotics have demonstrated to be able to lower blood cholesterol levels by different mechanisms, such as breaking down of prebiotic fibers to produce short-chain fatty acids in the gut, which can further inhibit cholesterol synthesis, or by assimilation and entrapment of cholesterol molecules into their membranes, thus eliminating cholesterol from the human gut (95–97).There are several studies on *Lactobacillus,* and *Bifidobacterium* strains with cholesterol assimilation activity (98, 99), while there are only few papers regarding cholesterol assimilation

by *Pediococcus* (100, 101). The cholesterol-lowering activity of *P. acidilactici* IRZ12B could be associated to the presence of cholesterol assimilation-related genes, coding membrane-associated proteins that can adhere to the cholesterol molecule and introduce it inside their cell (102) Cholesterol assimilation levels by strains grown in MRS broth supplemented with 0.3% ox gall and 100µg/mL cholesterol are reported in Figure 2.3. Only *P. acidilactici* IRZ12A and *P. acidilactici* IRZ12B could considerably lower the cholesterol level after 24h of incubation, with a 21.29 and 31.89% removal, respectively. One-way ANOVA indicated that the percentage of cholesterol assimilation varied significantly ($p<0.001$) between these two strains. *Limosilactobacillus pontis*IRZ14B also revealed cholesterol-lowering potential; however, the amount of cholesterol reduced by this strain was around 5%, much less than the two *P. acidilactici* strains.

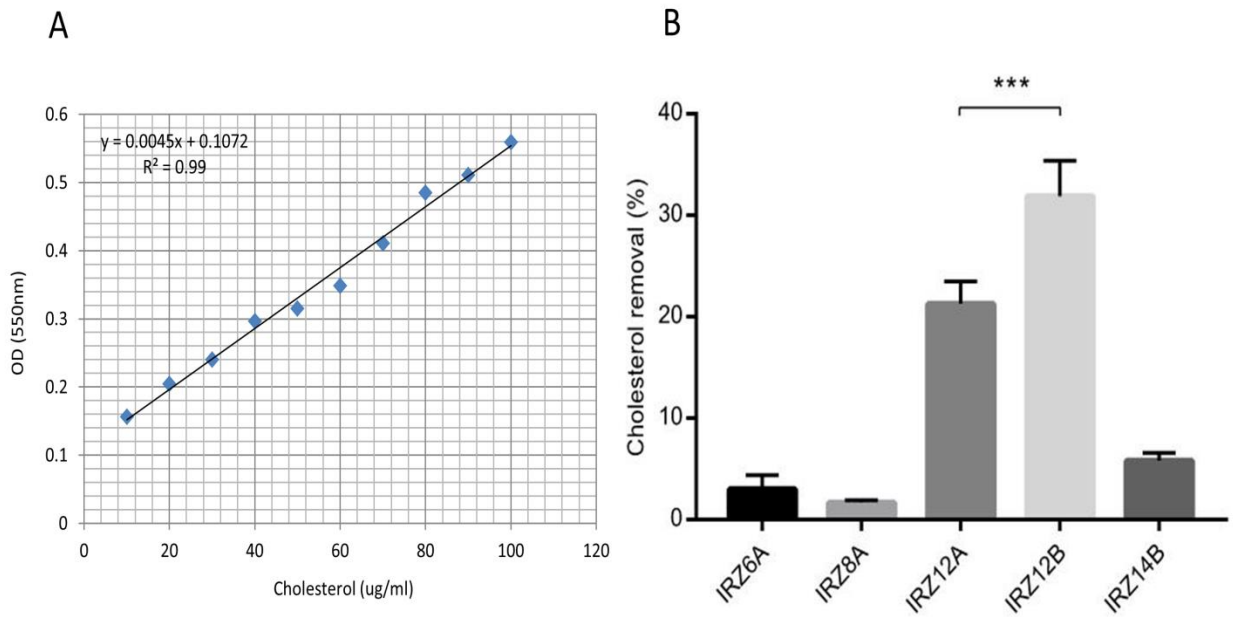

Figure 2.3. Cholesterol removal (%) of LAB strains incubated in MRS supplemented with 100 µg/mL cholesterol.

2.3.6. Screening for antimicrobial activity

The same five strains tested for cholesterol assimilation were also tested for the presence of antimicrobial activity against some selected bacteria. All strains produced a growth inhibition zone on plates (approximately 10mm radius of inhibition) towards *E. coli* DH1, *P. fluorescens* DSM50090, *S. xylosus* DSM20266, *B. subtilis* DSM10, *B. amyloliquefaciens* DSM7 and *L. innocua* DSM 20649. The same haloes were also present in the plates added with proteinase K, indicating that such inhibitory activity was not determined by a protein. Some *Pediococcus* strains are also known to produce a class of bacteriocins, named pediocins, antibacterial peptides belonging to the class II-a endowed with antimicrobial properties against Gram-positive bacteria (47). Indeed, *P. acidilactici* IRZ12B did not display bacteriocinogenic activity against the bacteria tested in the present study but was able to contrast the growth of some indicator strains, most probably because of its good pH lowering capability.

2.3.7. Genome sequencing, assembly, and annotation

Due to its very good capability to withstand gastrointestinal conditions and high ability to assimilate cholesterol, we decided to sequence the genome of strain IRZ12B to better examine the presence of potential features and/or problems. Nowadays, genome sequencing and analysis of new strains have become quite mandatory to define its exact taxonomy and to retrieve all the technological and safety-related information contained in its DNA (103, 104). The genome of the best cholesterol assimilating strain *P. acidilactici* IRZ12B was sequenced. The assembled genome produced 33 contigs (11 scaffolds), giving a genome size of 2.05 Mb with a GC content of 42.1%.The RAST server genome annotation showed a total number of 2064 predicted protein-coding sequences (CDSs) classified in 200 SEED subsystems .Fifty-six structural RNAs, and 16 genes related to different stress responses (osmotic, oxidative, and detoxification) were detected after the annotation too. It is worth mentioning that no virulence gene was detected from annotation. The largest section of the IRZ12B subsystem is allocated to the carbohydrate metabolism with 18.74% divided into different sub-classes as monosaccharides (29.37%), central carbohydrate metabolism (26.57%), di- and oligosaccharides (14.68%), fermentation (8.39%), amino sugars (6.99%), sugar alcohols (5.59%), organic acids (3.52%), one-carbon metabolism (2.79%), respectively.

The circular graphical genome map/annotation of P. acidilactici IRZ12B is shown in Figure 2.4. CDS on the forward and reverse strands, RNA genes, antimicrobial resistance genes (intrinsic), virulence factors, GC content, and GC skew are also reported in the map.

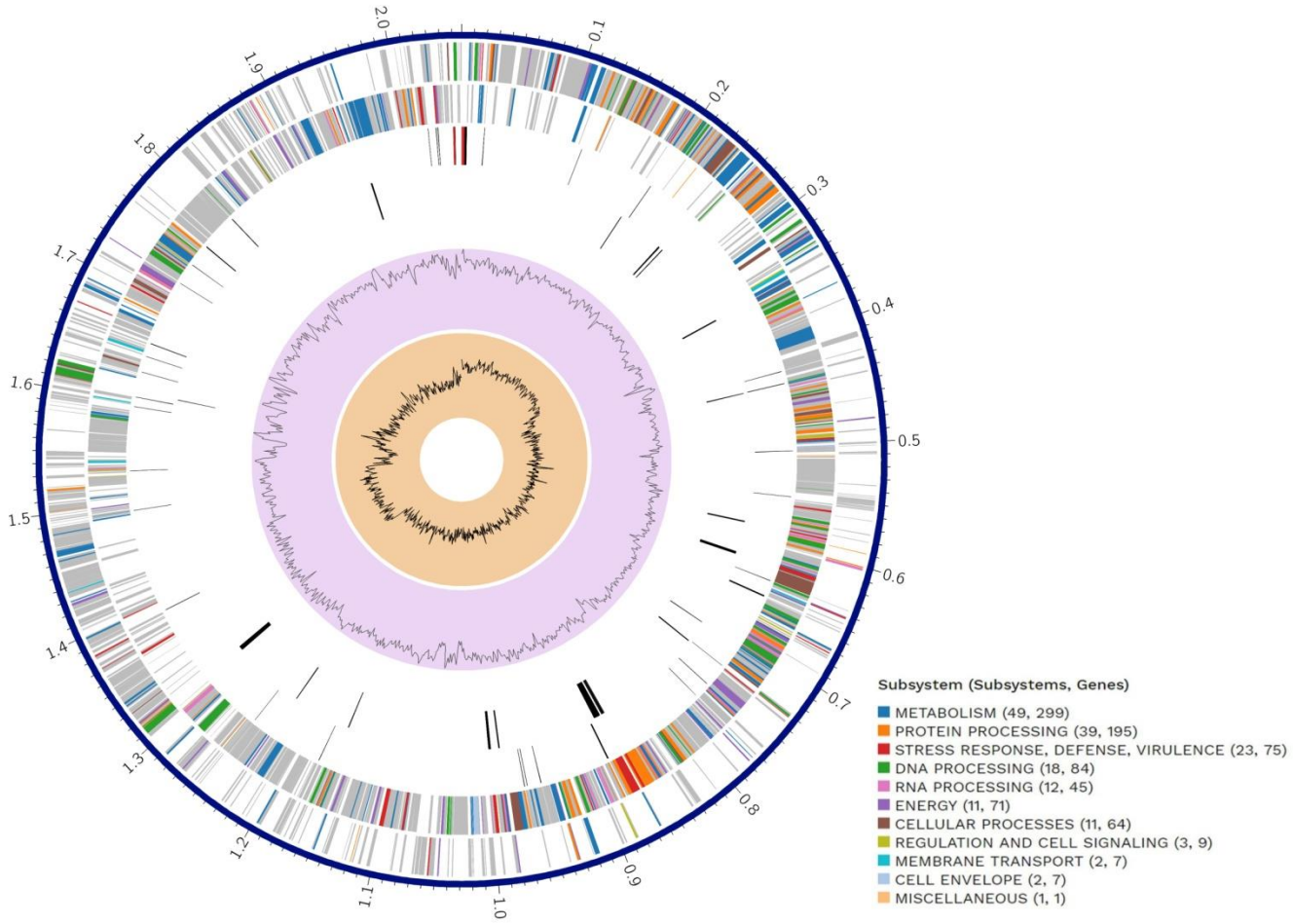

Figure 2.4. The circular graphical map includes, from outer to inner rings, CDS on the forward strand, CDS on the reverse strand, RNA genes, CDS with homology to known antimicrobial resistance genes, CDS with homology to known virulence factors, GC content, and GC skew. The colors of the CDS on the forward and reverse strands indicate the subsystem that these genes belong to.

2.3.8. Bioinformatical analysis and comparative genomics

The complete chromosome alignment by BLASTN comparison between the genome of IRZ12B and those of 29 different *Pediococcus* strains, including the type strain of each known species,

confirmed that this bacterium belongs to the species *P. acidilactici,* according to its position in the dendrogram reported in Figure 2.5.

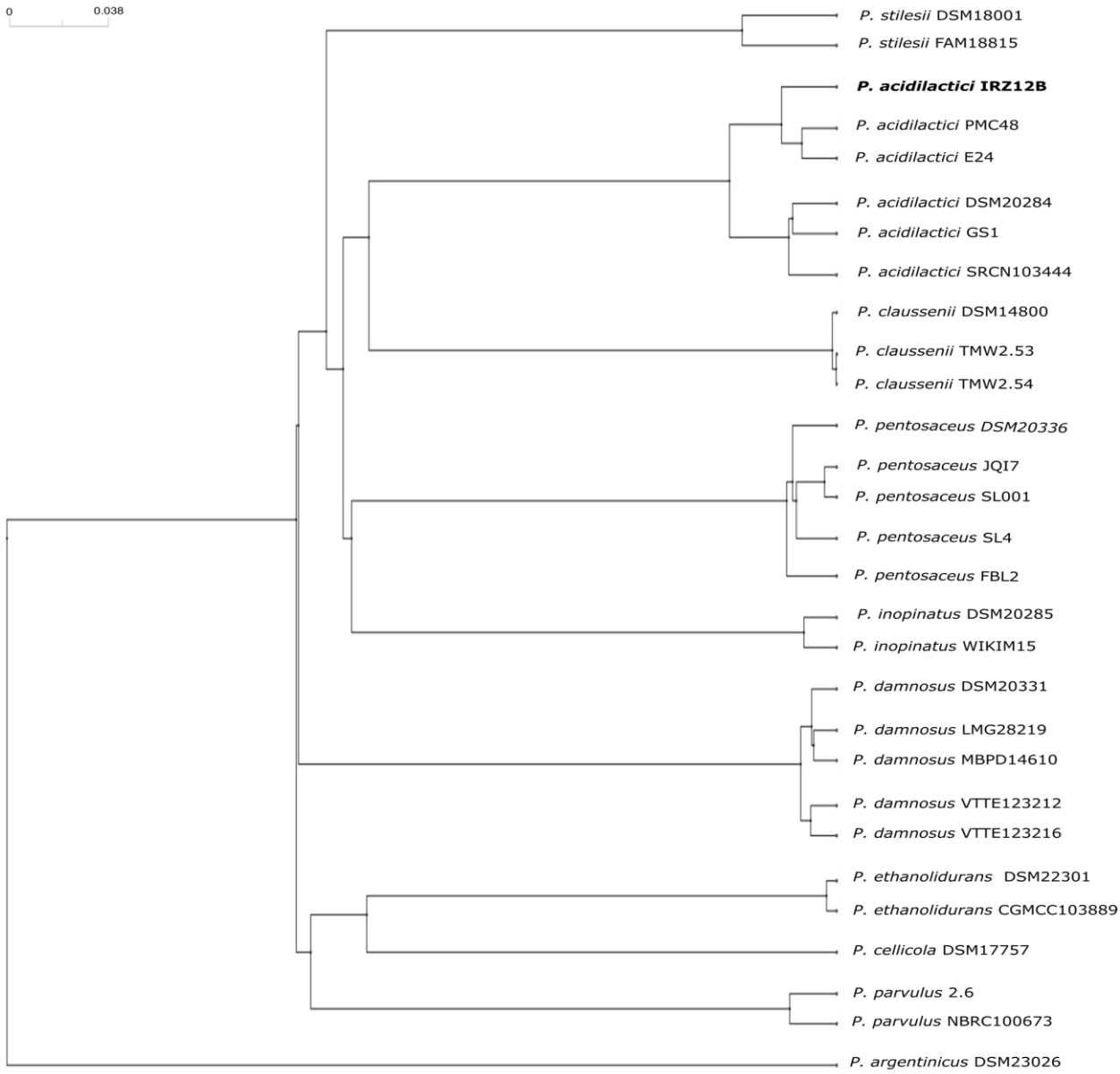

Figure2.5. Phylogenetic tree generated using the complete genomes of 28 different Pediococcus strains , including the type strain of each species along with *P. acidilactici* IRZ12B.

Pan/core-genome analysis using the Orthovenn server and the predicted amino acids from the six *P. acidilactici* genomes revealed that the core genome contains 1514 shared orthologous proteins (Figure 2.6A). All amino acid sequences were computed for their similarities using an e-value

cut-off of 1e−5 and an inflation value (−I) of 1.5 to define the orthologous cluster structure. The analysis detected a total of 2119 clusters with 628 orthologous clusters (at least contains two species). IRZ12B shares the largest number of orthologous proteins (1729) with *P. acidilactici* PMC48 (Figure 2.6B).

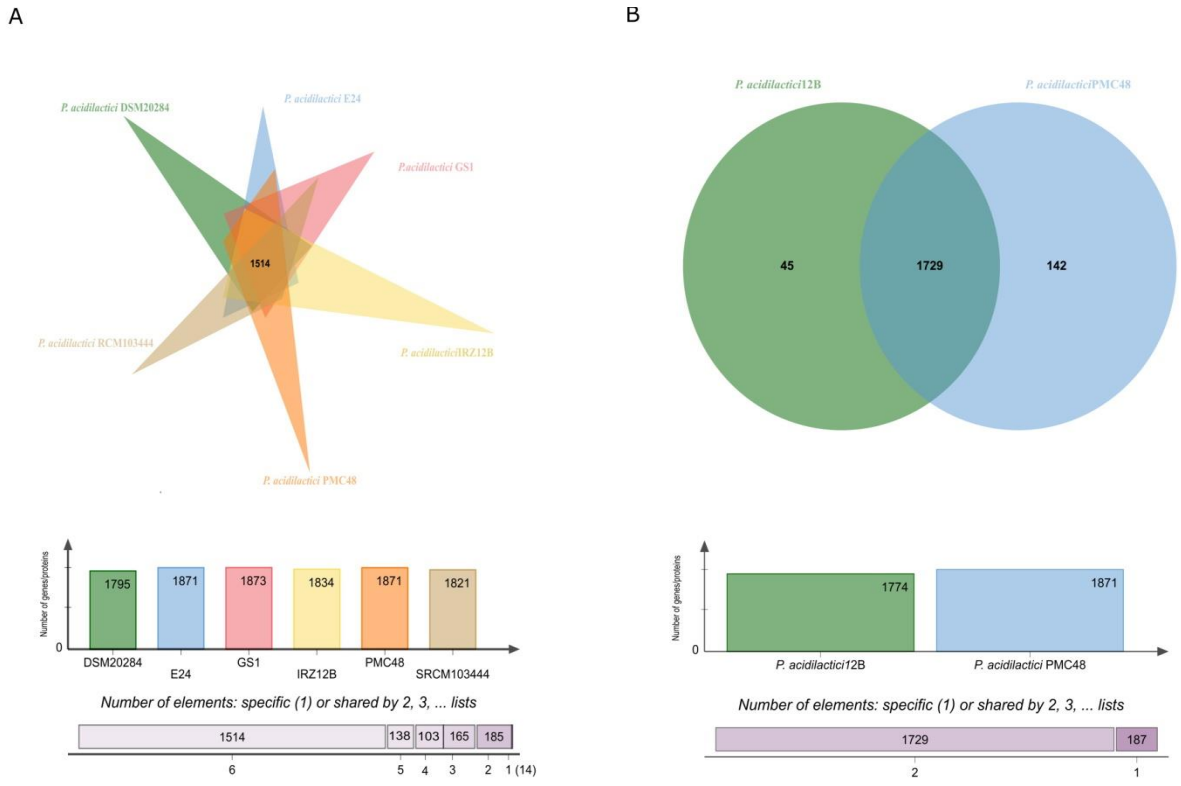

Figure 2.6. TheVenn diagram indicates the distribution of pan/core genes among *P. acidilactici* clusters. The number of clusters for each strain is also reported in the bars. The values of shared or single orthologous are shown in the purple boxes.

A deep search within the genome of *P. acidilactici*IRZ12B also found cholesterol assimilation-related genes, namely *ccpA, fba*, *lbpg_rs11190*, *lbpg_rs10085* (Table2.4). Moreover, the Res Finder and Plasmid Finder outcomes confirmed the absence of any acquired antibiotic resistance gene and plasmid sequences in *P. acidilactici*IRZ12B genome.

Table 2.4. Identification of cholesterol assimilation-related genes in *P. acidilactici* IRZ12B

Gene	Gene description	Organism	Identity (%)	Accession number
CcpA	Catabolite control protein A	*P. acidilactici* SRCM103367	99.80	CP035151.1
Fba	Class II fructose-1,6-bisphosphate aldolase	*P. acidilactici* BCC1	99.88	CP018763.1
LBPG_RS11190	FMN-binding protein	*P. acidilactici* PMC65	99.82	CP053421.1
LBPG_RS10085	MFS transporter	*P. acidilactici* HN9	99.18	CP061715.1

2.3.9. Plant selection screening

For the screening step, six different medicinal plants, namely *Malva*, *Calendula*, *Echinacea*, Olivo (olive tree), Vite (grapevine), Santoreggia, were fermented with *P. acidilactici* IRZ12B, a potential probiotic strain. After the fermentation step, the outcome revealed that *P. acidilactici* IRZ12B could effectively ferment *Malva*, *Calendula*, *Echinacea* but not Olivo, Vite and Santoreggia, which could be due to the high nutritional value of *Malva*, *Calendula*, and *Echinacea*. (Figure 2.7)(103) (104), (105) .According to the result, *Malva*, *Calendula*, and *Echinacea* were selected for the fermentation by *P. acidilactici* IRZ12B.

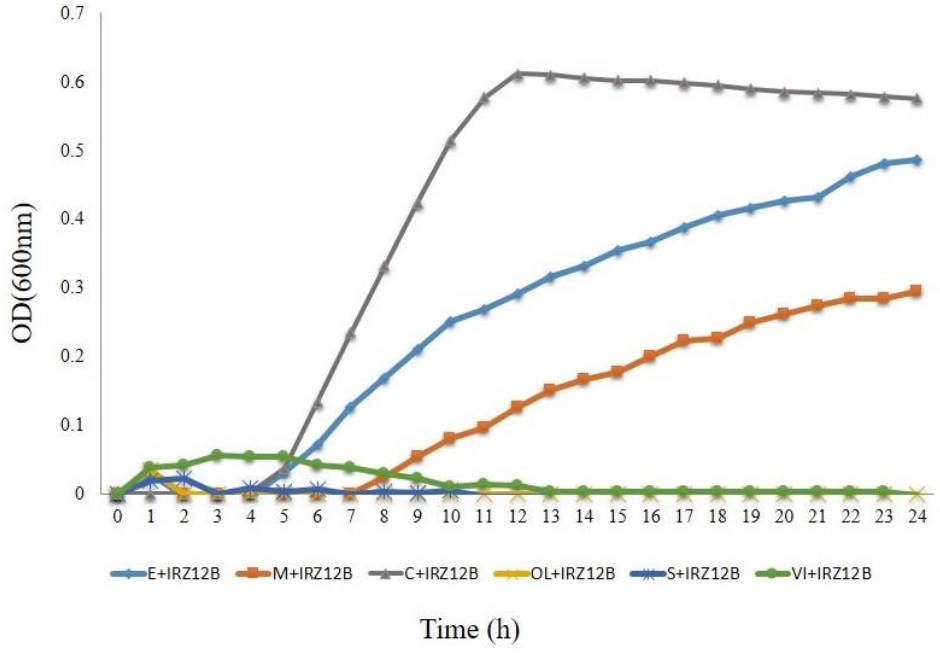

Figure 2.7. *P. acidilactici* IRZ12B growth curve on *Malva, Calendula, Echinacea*, Olivo, Vite, and Santoreggia.

2.3.10. pH measurement before and after fermentation

The pH before and after fermentation of three selected plants was recorded, and the outcome showed that *P. acidilactici* IRZ12B strain could acidify the *Calendula* significantly ($p \leq 0.05$) after the fermentation (Figure 2.8). We detected a slightly lower initial pH value for non-fermented *Calendula* (5.20) compared to *Echinacea* (5.46) and *Malva* (5.74). It is worth mentioning that similar pH values (no significant difference) have been recorded for all non-fermented herbal media before and after incubation. Usually, the LAB strains produce lactic acid from different carbohydrates, which result in pH reduction (106)

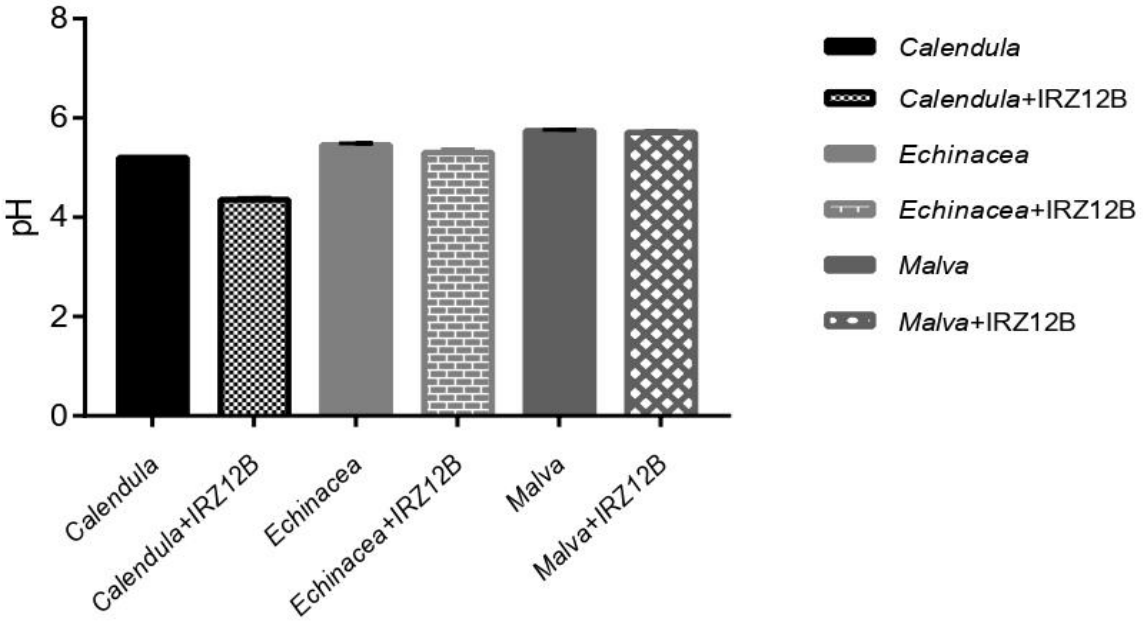

Figure 2.8. pH value recorded before and after fermentation for *Calendula*, *Malva* and *Echinacea* with *P. acidilactici* IRZ12B

2.3.11. Bacterial enumeration

The bacterial population count was recorded before and after fermentation in all combinations. We have detected more than 1.2 logarithmic growth, which proves the high nutritional values of these three plants. The bacteria used in this study are all fastidious and require different kinds of vitamins and amino acids to grow. The highest growth has been detected in *Calendula*+ IRZ12B with a 2.3 log growth (Figure 2.9).

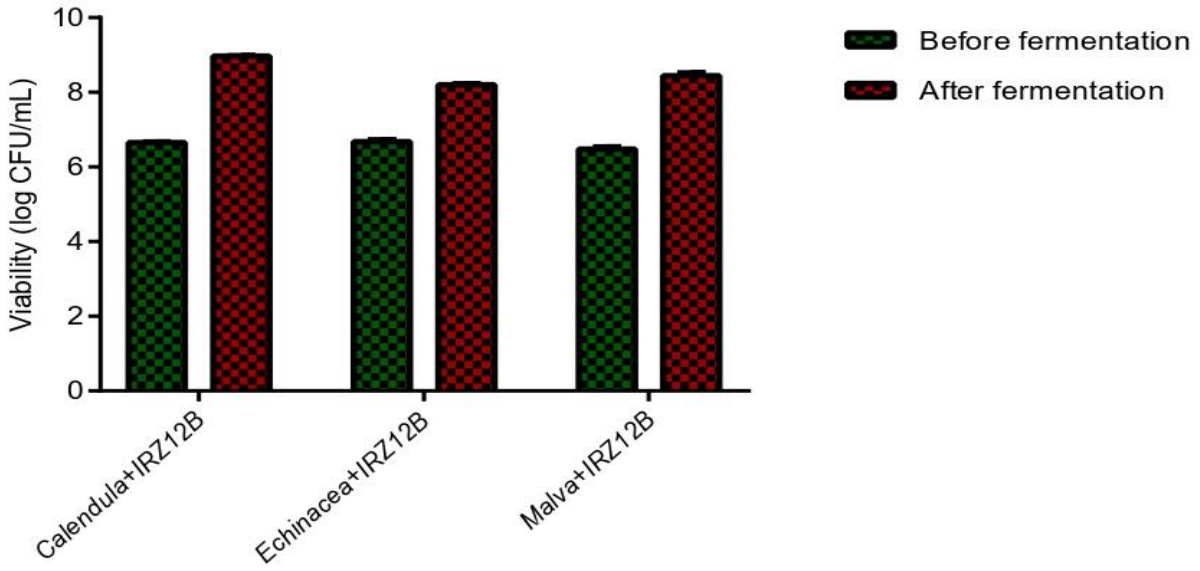

Figure 2.9. *P. acidilactici* IRZ12B enumeration (log CFU/mL) before and after fermentation on *Calendula*, *Malva,* and *Echinacea*.

2.3.12. Cytotoxicity effect on intestinal cell lines

Cytotoxicity effects of the fermented/non-fermented plants on differentiated human intestinal cells were evaluated using different concentrations (25, 50, 100, 200, and 500 µg/mL). The results indicated no significant viability reduction when the cells were treated with different concentrations of no-fermented/ fermented plants compared with the control (DMEM). Therefore, we could consider them as non-toxic to healthy intestinal cells (Figure 2.10). Using officinal plants as natural medicine is widespread worldwide, and their history dates back to ancient people. One of the main concerns about medicinal plants is their safety assessments on human health, whether they can affect the cell viability of healthy human cells or not. Differentiated Caco2 cells were isolated in 1977 from the human intestinal cell line (107). From 1977 onward, this type of cell has been considered for different *in vitro* studies (108). Interestingly, many studies have proved that medicinal herbs do not possess any toxicity against differentiated Caco2 cells (healthy cells), while they can negatively affect carcinogenic Caco2 cells. (109).

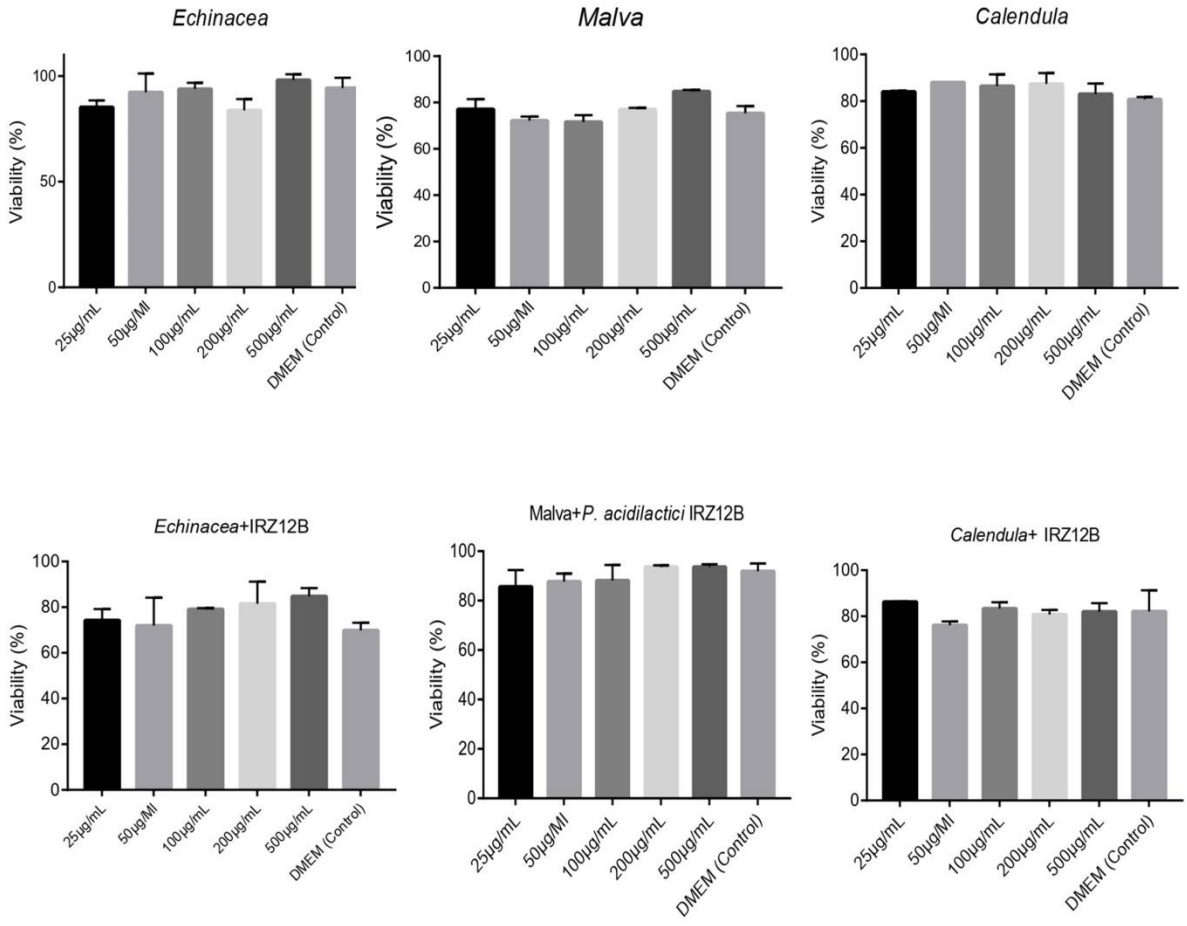

Figure 2.10. Differentiated Caco2 cell viability after treatment with fermented *Calendula, Malva,* and *Echinacea*.

2.3.13. Cytotoxicity effect on Human Breast cancer cells (MCF -7)

The cytotoxicity effect of the fermented/non-fermented plants was carried out using different concentrations. The outcome showed that the fermented *Calendula* with potential the probiotic strain IRZ12B could significantly inhibit the growth of cancer cells (Figure 2.11). A breast cancer cell line (MCF-7) was isolated for the first time in 1970 from a 69-year-old woman at the Michigan Cancer Foundation (110). From that time onward, many scientists worked on MCF-7 cells to find ways to treat breast cancer cells and deeply study the pathways and reasons behind them.

The effects of natural compounds on cancer cells are related to suppressing initiation, promotion, or progression of tumours. Dietary phytochemicals have an anti-cancer impact on different

molecular mechanisms and depend on their action by using various molecular targets. They have different pathways on oncogenic signalling (111). As reported in other studies, a variety of bioactive compounds present in *Calendula* can inhibit the growth of cancer cells (112). MicroRNAs (miRNA), a small single-stranded non-coding RNA molecule, may function as tumor suppressors or oncogenes in a particular situation (113). Dysregulated miRNA has critical effects on cancer indications such as resisting cell death, activating invasion and metastasis, sustaining proliferative signalling, evading growth suppressors, and inducing angiogenesis (114). To control the rapid proliferation of cancer cells, the initiation of apoptosis or cell death is essential. Some studies showed that *BCL-2* is a crucial regulator of apoptosis, and its overexpression can promote the stability of breast cancer cells (115). On the other side, studies indicated that some miRNAs, such as miR-21, can target the *BCL-2* gene transcription and increase the MCF-7 cells apoptosis (116).

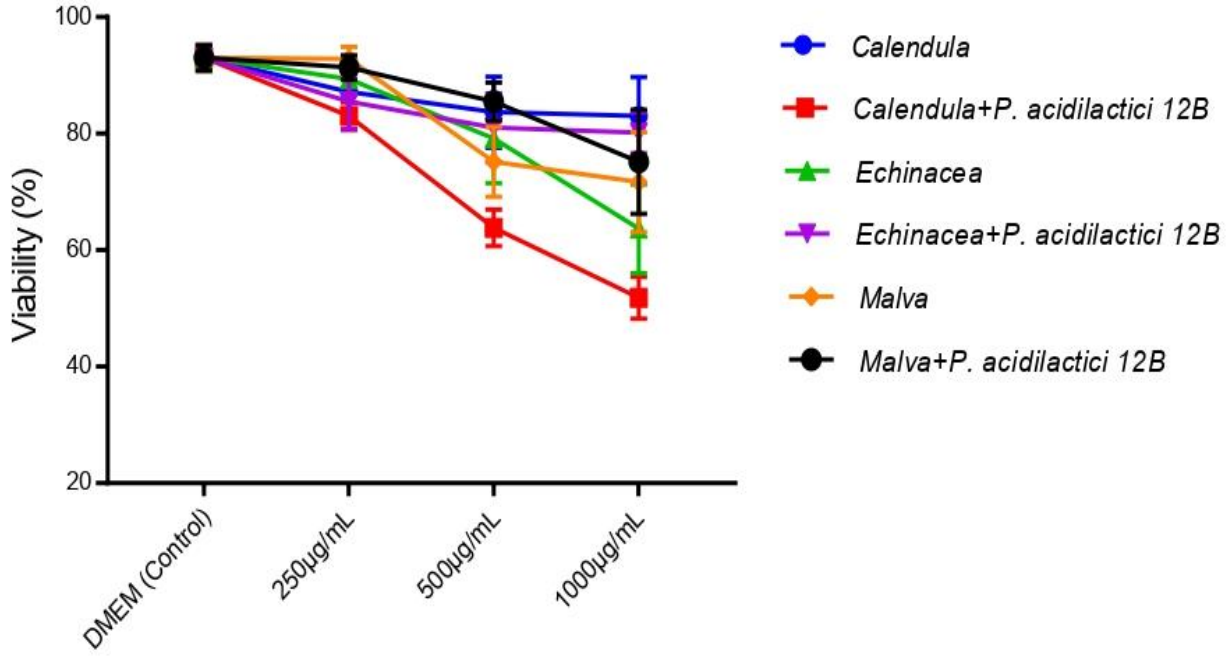

Figure 2.11. MCF-7 cells viability after treatment with fermented/non-fermented *Calendula*, *Malva,* and *Echinacea***.**

2.4. Conclusion

The genomic study conducted on *P. acidilactici* IRZ12B revealed the absence of negative characteristics such as virulence genes, transmissible antibiotic gene resistance, and plasmids. Moreover, functional food preparation using three officinal plants, *Malva*, *Calendula*, and *Echinacea* fermented with *P. acidilactici* IRZ12B revealed that fermented herbs with the probiotic strain did not have any cytotoxic effect on healthy human intestinal cell line (differentiated Caco-2); however, they could reduce cell viability on breast cancer cells (MCF-7) significantly. There results from genetic analyses, together with the outcomes of the phenotypic tests, make *P. acidilactici* IRZ12B a promising probiotic candidate to be considered for its possible use in the production of novel functional foods.

Chapter 3

Effects of 2-fucosyllactose-based encapsulation on probiotic properties in *Streptococcus thermophilus*

Abstract

Streptococcus thermophilus is widely used in dairy fermentation as a starter culture for yogurt and cheese production. Many strains are endowed with potential probiotic properties; however, since they might not survive in adequate amounts after transit through the human gastrointestinal tract, it is advisable to improve cell survivability during this passage. The present study evaluates the use of 2′-fucosyllactose, a prebiotic molecule from human milk, compared with other known molecules, such as gelatin and inulin, to form alginate-based microcapsules to fulfil these requirements. Such microcapsules, obtained by the extrusion technique, were evaluated in terms of encapsulation efficiency, storage stability, gastrointestinal condition resistance, and cell release kinetics. Results reveal that microcapsules made using 2′-fucosyllactose and those with inulin resulted in the most efficient structure to protect *S. thermophilus* strain TH982 under simulated gastrointestinal conditions (less than 0.45 log CFU/g decrease for both agents). In addition, a prompt and abundant release of encapsulated cells was detected after only 30 min from microcapsules made with sodium alginate plus 2′-fucosyllactose in simulated gastrointestinal fluid (more than 90% of the cells). These encouraging results represent the first report on the effects of 2′-fucosyllactose used as a co-encapsulating agent.

3.1. Introduction

The genus *Streptococcus* includes more than 70 species, but to date, only *S. thermophilus* possesses the GRAS (generally recognized as safe) status due to its long history of safe application in food production (34) For this reason, this is the only species of the genus used in the food industry and represents the second most important species of industrial lactic acid bacteria (LAB) after *Lactococcus lactis* and one of the primary starters of yogurt (117, 118) .Furthermore, many *S. thermophilus* strains have several technological properties, such as sugar metabolism, galactose utilization capability, proteolytic activity, and urease activity (119).

Some strains have also shown potential as a probiotic, having revealed various health benefits (120, 121). *S. thermophilus* is a bacterium of non-human origin and, although known to be sensitive to the acidic gastric conditions, it was able to survive the gastrointestinal tract and moderately capable of adhesion to intestinal epithelial cells (122), thus making it a transient

probiotic (122, 123). Nowadays, probiotics are one of the driving forces in the design of functional foods, particularly dairy products; hence it is advisable to improve cell survivability during the gastrointestinal passage. The inclusion of probiotics into microcapsules seems to be a promising solution and also a means to control the release of cells into the target tissues (124) .

Several techniques are available for encapsulation, which differ on the nature of their core (content) and intended use (125). Encapsulation techniques applied to the food industry include spray-drying, fluid bed coating, spray-chilling or spray cooling, extrusion, emulsification, coacervation, co-extrusion, inclusion complexation, liposome entrapment, centrifugal extrusion, encapsulation by a rapid expansion of supercritical fluid (RESS), freeze- or vacuum drying, and nanotechnological approaches (126). The use of the extrusion technology has many advantages for the encapsulation of probiotic cells. It is a simple and inexpensive method using mild operations, and the procedure does not involve any harmful solvent. Moreover, this technique appears to promote probiotic cell viability (127–129). Combining alginate with some prebiotic molecules can also enhance cell protection in food systems (125). Prebiotics form three-dimensional microcrystal networks that interact together, forming small aggregates that contribute to the better protection of the cells (130) .The most frequently used prebiotics in co-encapsulation are oligosaccharides, inulin, and resistant starches (130). 2'-fucosyllactose (2′-FL), is a neutral trisaccharide composed of L-fucose, D-galactose, and D-glucose and is the most abundant oligosaccharide in human milk (HMO), accounting for about 30% of all of HMOs, i.e., 2–3 g/L (131). This prebiotic molecule has been reported to display numerous health advantages in humans (132–134) Another attractive characteristic of 2′-FL is its simplicity, which facilitates its de novo synthesis using microbial, enzymatic, or chemical methods (135). In this study, we aimed at evaluating the protective effect of 2'-FL on *S. thermophilus* TH982 cells, a potential probiotic with the ability to produce high levels of folate (136) compared to some other common co-encapsulating materials in alginate beads.

3.2. Materials and Methods

3.2.1. Bacteria and growth conditions

S. thermophilus TH982 (137) is part of the collection of the Department of Agronomy, Food, Natural resources, Animals and Environment, University of Padova, Italy. *S. thermophilus* TH982 was stored at 80 °C in M17 broth (Sigma-Aldrich, St. Louis, MI, USA) containing glycerol (25% v/v) and was activated before each experiment by culturing it in M17 (Sigma-Aldrich) broth at 37 °C for 24 h.

3.2.2. Encapsulation procedure

The components used for encapsulation of *S. thermophilus* TH982 were sodium alginate (S, Sigma-Aldrich), gelatin (G, from bovine skin, Sigma-Aldrich), inulin (I, from chicory, Sigma-Aldrich) and 2′-fucosyllactose (F, Sigma-Aldrich). Fifty milliliters of reactivated cells in M17 broth were centrifuged (Hettich, Westphalia, Germany) at 5500 rpm for 5 min. The bacterial pellets were washed twice with sterile *saline solution* (0.85%) (Sigma-Aldrich) and added to the encapsulation matrix. The encapsulating matrix was prepared using the extrusion technique through the combination of sodium alginate with either inulin, gelatin, or 2′-fucosyllactose, each one at a concentration of 2% (*w*/*v*). Sodium alginate alone was also used as a control. The different matrices were mixed and added to the *S. thermophilus* TH982 pellet suspension.

The mixture was then injected using a sterile syringe with a 450 µm needle into sterile 0.3 M $CaCl_2$ (Sigma-Aldrich) hardener solution. The distance from the bottom of the nozzle and the surface of the $CaCl_2$ solution was kept at 10 cm (Figure3.1). The microcapsules were left in the hardening solution ($CaCl_2$) for 30 min at room temperature. Finally, capsules were transferred in 0.1% (*w*/*v*) sterile peptone water solution and stored at 4 °C until further use. The morphology of the microcapsules was examined using an optical stereomicroscope (Olympus, Tokyo, Japan).

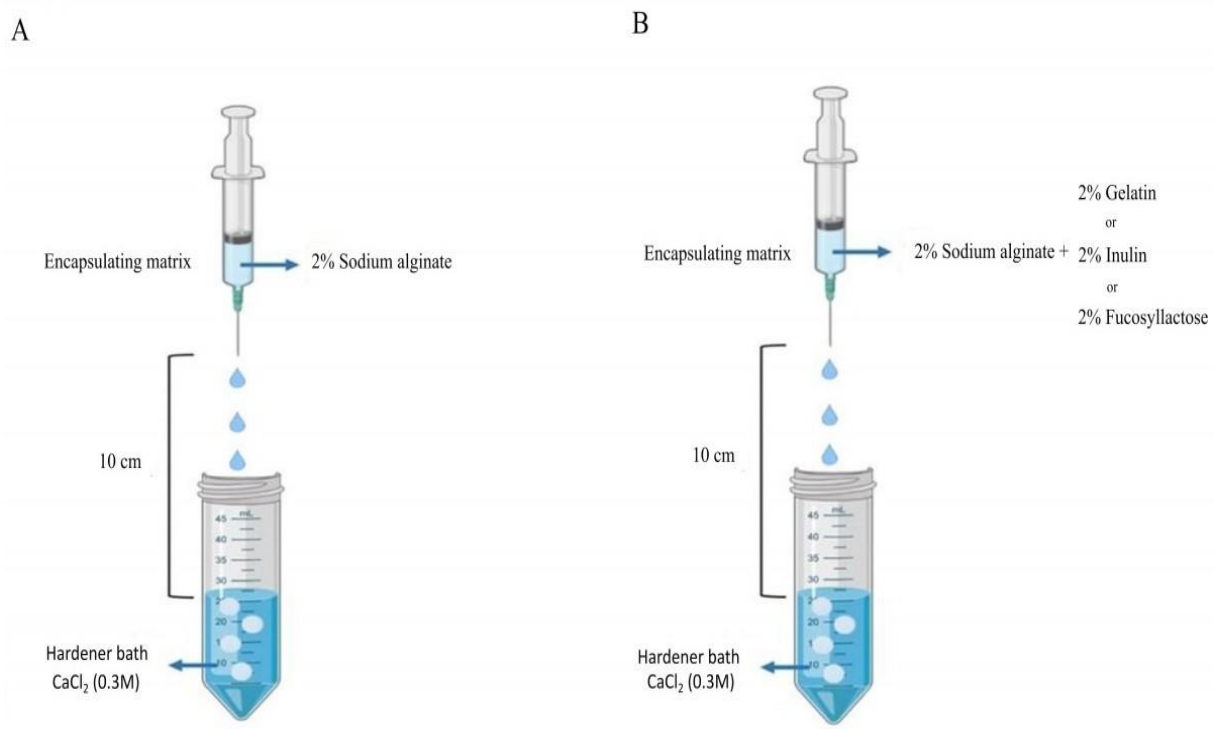

Figure 3.1. Scheme of microencapsulation procedure of *S. thermophilus* TH982 cells using the extrusion technique. (**A**) The sodium alginate alone as control. (**B**) Sodium alginate with other agents.

3.2.3. Encapsulation Efficiency

One milliliter of each type of capsule solution was added to 9 mL of *saline solution* (0.85%), serially diluted, and plated on M17 agar medium to determine the viable cell concentration in capsule solutions. One gram of each type of capsule was homogenized in 9 mL of 10% (*w*/*v*) sterilized sodium citrate by vortexing for 1 min, serially diluted, and plated on M17 to get the number of entrapped cells per g of capsule. The enumeration of viable cells from both the solution and the capsule was done using the micro drop technique by placing 20 µL aliquots on the surface of M17 agar plates and incubating at 37 ˚C for 48 h. The encapsulation efficiency (EE%) was determined using the equation (138)

$$EE\% = (N_1/N_0) \times 100$$

where N_1 is the number of viable entrapped cells (log CFU/g) released after the encapsulation procedure and N_0 is the number of viable cells (log CFU/mL) added to the mixture before encapsulation.

3.2.4. Storage stability in skimmed milk

One gram of microcapsules was suspended in 9 mL of 10% (*w*/*v*) reconstituted skimmed milk, stored under refrigerated conditions (4 ˚C) and sampled at different time intervals, namely after 0, 7, 14 and 21 days to evaluate the survival of encapsulated *S. thermophilus* TH982 (139).The survival of free (non-encapsulated) bacterial cells was determined by adding 9 mL of 10% (*w*/*v*) reconstituted skimmed milk (Sigma-Aldrich) to 1 mL of free cells as well. At each time interval, 1 g aliquot of capsules was aseptically centrifuged at 5500 rpm for 5 min. The supernatant was discarded and the cells were allowed to release in 9 mL of 10% (*w*/*v*) sodium citrate by vortexing for 1 min, serially diluted, and plated on M17 agar, followed by incubation at 37 ˚C for 48 h. The survival was determined as the number of cells recovered during different storage time intervals with respect to the number of initial entrapped cells.

3.2.5. Survivability under simulated gastrointestinal conditions

The simulated gastrointestinal conditions were obtained using basic fluid, gastric fluid, and intestinal fluid. The basic fluid was prepared by dissolving (per liter): 1.12 g potassium chloride (Sigma-Aldrich), 2.0 g sodium chloride (Sigma-Aldrich), 0.11 g calcium chloride (Sigma-Aldrich), and 0.4 g potassium dihydrogen phosphate (Sigma-Aldrich) in *saline solution* (0.85%). The basic fluid was sterilized by autoclaving at 121 ˚C for 15 min. The simulated gastric fluid (SGJ) and simulated intestinal fluid (SIJ) were prepared based on a modified method published by Singh et al. (139). The gastric fluid consisted of 0.01 g/L of swine pepsin (Sigma-Aldrich) and 0.01 g/L of swine mucin (Sigma-Aldrich) added directly to the sterile basic juice. The pH was adjusted to 2.5 with 1 N HCl, and the liquid was filter-sterilized using a 0.22 µm membrane filter (Sigma-Aldrich). The intestinal fluid contained (per liter of basic fluid): 0.01 g pancreatin (Sigma- Aldrich), 0.08 g Ox-bile extract (Sigma-Aldrich), and 0.01 g lysozyme (Sigma-Aldrich). The pH was adjusted to 7.5 with 1 N NaOH, and the juice was filter sterilized. Both gastric and intestinal fluids were prepared fresh on the day of the experiment. Overnight bacterial cultures were obtained after subculturing in M17 broth, and capsules were prepared as described in the encapsulation procedure. One gram of each type of capsule was added to 9 mL of SGJ and incubated at 37 ˚C for 1 h

to evaluate the survivability of *S. thermophilus* TH982 under gastric conditions. For the survival of *S. thermophilus* TH982 under gastrointestinal conditions, 9 mL of SIJ were added following gastric fluid incubation, and the capsules and free cells with simulated gastrointestinal fluid were left at 37 °C for a further 2 h. The cell viability was evaluated for free and encapsulated *S. thermophilus* after gastric and gastrointestinal incubations by plating on M17 agar and using the micro drop technique, followed by incubation at 37 °C for 48 h.

3.2.6. Release kinetics

The release kinetics from capsules were determined using phosphate solution without enzymes. The release of encapsulated *S. thermophilus* TH982 into the medium was evaluated according to Shi et al. (140) with some modifications. A phosphate solution (6.8 g/L of K_2HPO_4, 50 mM) without the addition of intestinal enzymes was prepared, the pH was adjusted to 7.5 with 1 N HCl and subsequently sterilized by autoclaving at 121 °C for 15 min. Microcapsules (1 g) from different matrices were added to 9 mL of the abovementioned phosphate solution and incubated at 37 °C with agitation (100 rpm). At predetermined time intervals (0, 30, 60, 90, 120, 150, and 180 min), 100 µL of solution were taken from each capsule type, serially diluted, and plated using the micro drop technique on M17 agar to detect the number of released cells. The number of released cells was determined after 48 h of incubation at 37 °C and expressed as log CFU/mL of K_2HPO_4 solution.

3.2.7. Statistical analysis

All the experiments were performed in triplicate. Data were analyzed using a one- way analysis of variance (ANOVA). Tukey's test was used as a post-hoc analysis by the GraphPad Prism software (version 7, GraphPad Software, Inc., San Diego, CA, USA). Results were considered significantly different for p values lower than 0.05.

3.3. Results and Discussion

3.3.1. Morphology and encapsulation efficiency

All capsules appeared globular and irregular in shape with a rough surface and a drop-like shape. This result is probably due to the high surface tension of the used hardening solution ($CaCl_2$) that determines an imperfect sphere formation (141) .However, no surface cavities or fractures were visible. The average size of capsules was 3.5 0.52 (mm) without any significant difference among the different microcapsules types. The capsules with S + G showed a more dense structure, as confirmed by the viscosity of G solution, due to electrostatic interactions between amino groups of G and carboxyl groups of S, which make cells more resistant to enzymatic and acidic hydrolysis (142). Encapsulation efficiencies (EE%) of the four microcapsule types are reported in Table 1. EE% is a parameter that describes both the survival of viable cells and the efficacy of entrapment of the encapsulation procedure (143) .The yield of *S. thermophilus* TH982 viable cells co-encapsulated using S + F (96.13%) was significantly higher ($p < 0.01$) than that of cells encapsulated with S alone (91.07%), used as control, and was similar (not statistically different) to S + I (98.61%). The high EE% obtained with S + F and S + I matrices might be due to the decrease in the porosity of the gel-beads and, consequently, to a reduction in the leakage of entrapped *S. thermophilus* TH982 cells (144). Besides, incorporating prebiotics as material for encapsulation may better protect probiotics in food systems and inside the gastrointestinal tract due to mutual cells/prebiotic interactions (145) .By contrast, the number of viable cells encapsulated in S alone and S + G showed no significant difference (EE% 91.07 and 90.50, respectively).The main reason why EE% is normally lower than 100% is linked to cell damage due to detrimental conditions caused by the encapsulation process itself, such as shear stress and the use of concentrated solutions. Furthermore, during the time required for the hardening of capsules, a physical loss of cells can occur in significant numbers (143) It should also be noted that a dissolution process is required to determine the number of viable cells in the microcapsules, and therefore an incomplete disintegration can underestimate the calculated EE% value. In particular, for the solubilization of S-based capsules, sodium citrate (10% w/v) was used, which is a chelating agent (144) that can remove calcium from the “egg-box” structure, leading to the destabilization of alginate coating and to the effective release of cells (146)

Table 3.1. Encapsulation efficiency (EE%) of S. thermophilus TH982 using the different encapsulating matri*ces*. Values are the mean ± standard deviation (SD) of triplicate experiments (n=3).

Capsule formulation	Encapsulation solution (log CFU/ml)	After encapsulation (log CFU/g)	Encapsulation efficiency (EE%)
S	9.46±0.04	8.61±0.19	91.07[a]
S+I	9.17±0.21	9.04±0.12	98.61[b]
S+G	9.39±0.09	8.50±0.04	90.50[a]
S+F	9.34±0.03	8.97±0.07	96.13[b]

[a, b] indicates statistically significant (P <0.05) differences between different formulations.

3.3.2. Storage stability in skimmed milk

In order to test the viability of *S. thermophilus* cells inside capsules during prolonged storage, we maintained them in skimmed milk at refrigeration conditions (4 ˚C) for 21 days (Figure 3.2). At the end of the period, cell viability showed 0.46, 1.62, 1.79, and 1.97 log decrease for *S. thermophilus* TH982 cells encapsulated with S + G, S + F, S, and S + I, respectively. Capsules containing G as a co-encapsulating agent showed a better performance after 21 days with respect to other co-encapsulating agents. However, data show that free cells had the highest viability level throughout the storage period since there was no significant difference between initial (8.90 0.08 log CFU/mL; day0) and final (8.94 0.05 log CFU/mL; day21) values. Interestingly, S, S + I, and S + F capsules revealed a similar, not significantly different, viability reduction ($p < 0.05$), differently from some studies that indicate an increase in survivability during refrigerated storage conditions, which could be linked to the difference in species/strain used. *S. thermophilus* is frequently used in the production of many dairy products, and it is highly adapted to the dairy environment. It was reported that encapsulation improved the stability of *L. plantarum* during storage (147) , and encapsulation of bacteria in alginate was found to improve survivability when compared to unprotected cell counts stored in skimmed milk for 24 h (129) .Since FAO and the International Dairy Federation (IDF) recommend that the minimum content of probiotic cells should be 10^7 CFU/g of product at the time of consumption (148), the four encapsulating agents used in this study

were efficient in maintaining the required viability of *S. thermophilus* TH982 after refrigerated storage in skimmed milk. The high numbers suggested by IDF have been proposed to compensate for the possible numerical reduction of probiotic organisms during passage through the human stomach and intestine. Cells must remain viable throughout the projected shelf-life period of a product so that when consumed, the product contains a sufficient number of viable cells.

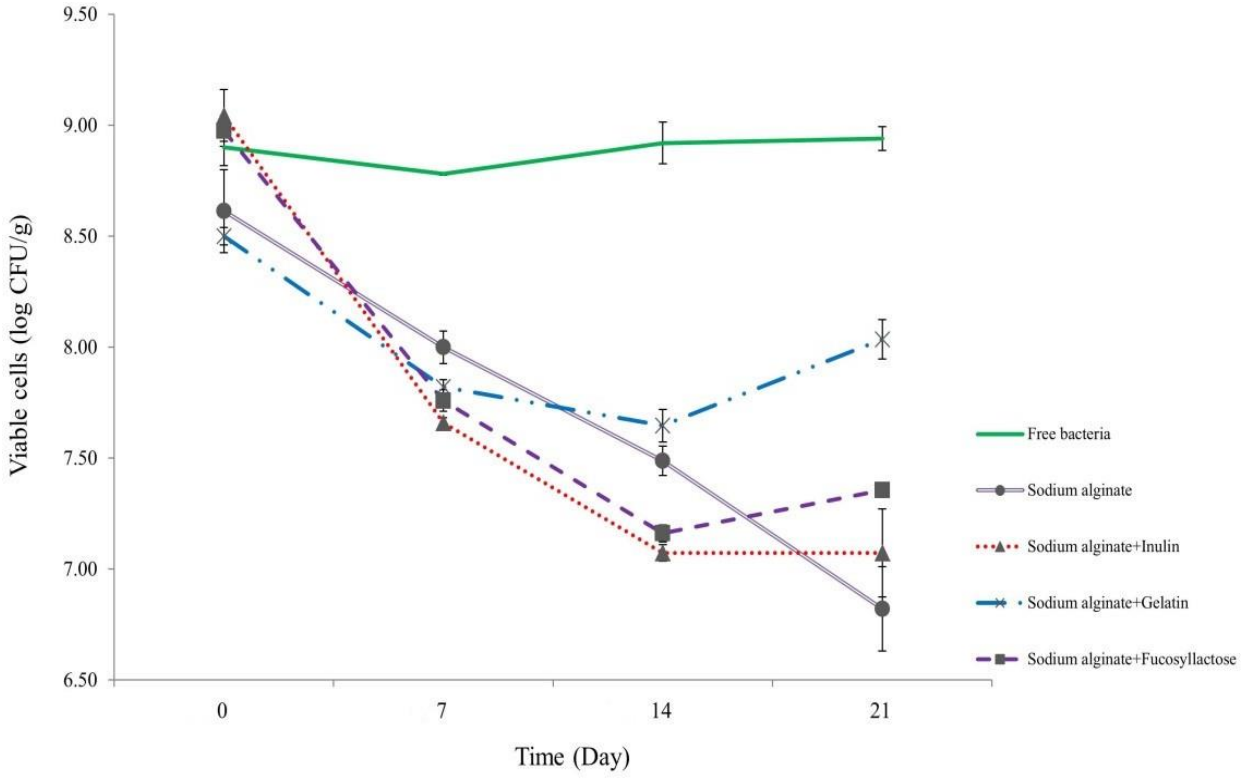

Figure 3.2. Survivability of encapsulated and free *S. thermophilus* TH982 cells during 21 days of storage in skimmed milk at 4 ˚C.

The viability of probiotic bacteria in food products is affected by many intrinsic and extrinsic aspects such as dissolved oxygen and oxygen permeation through the package, post acidification in fermented products (lactic and acetic acids), pH, storage and incubation temperature, duration of fermentation, production of hydrogen peroxide due to bacterial metabolism, and processing conditions (149, 150). Moreover, the specific strains used, the

interaction between species, availability of nutrients, and growth promoters and inhibitors can affect cells survivability.

The great survivability of free *S. thermophilus* TH982 cells in skimmed milk is probably due to the fact that *S. thermophilus* is a species highly adapted to grow on lactose as its energy and carbon source, that is internalized by the lactose permease (LacS) and hydrolyzed to glucose and galactose by β-galactosidase (LacZ) (151). It is likely that the entrapment of *S. thermophilus* TH982 cells into capsules of different matrices probably led to a reduced diffusion of lactose inside the capsule layer or membrane, limiting the possibility of its use by the entrapped cells (149).

3.3.3. Survivability under simulated gastrointestinal conditions

The survival of probiotics under gastrointestinal conditions represents a significant issue for their effectiveness, and microencapsulation could represent a valid method to provide significant protection .The survival of free and encapsulated *S. thermophilus* TH982 cells after 1, 2 and 3 h of incubation in simulated gastrointestinal conditions are presented in Figure 3.3. After 1 h of gastric fluid incubation, the highest reduction of viable cells was found, as expected, for free *S. thermophilus* TH982, as the initial number of 10.40 ± 0.10 log CFU/mL was diminished by 0.95 log CFU/mL. A notable decrease in viability after 3 h of gastrointestinal conditions was confirmed for free *S. thermophilus* TH982 cells, reduced by approximately 1.60-log CFU/mL, dropping to 8.79 ± 0.02 log CFU/mL. By contrast, for cells encapsulated with S alone, only 1.05 log CFU/g reduction of viable cells was observed, implying that microencapsulation provided protection compared to the free cells. This protection offered by sodium alginate might be related to the establishment of a hydrogel barrier through an external layer of sodium alginate that has retarded the permeation of simulated fluids into the capsules and thus the interaction with the probiotic cells (152) .

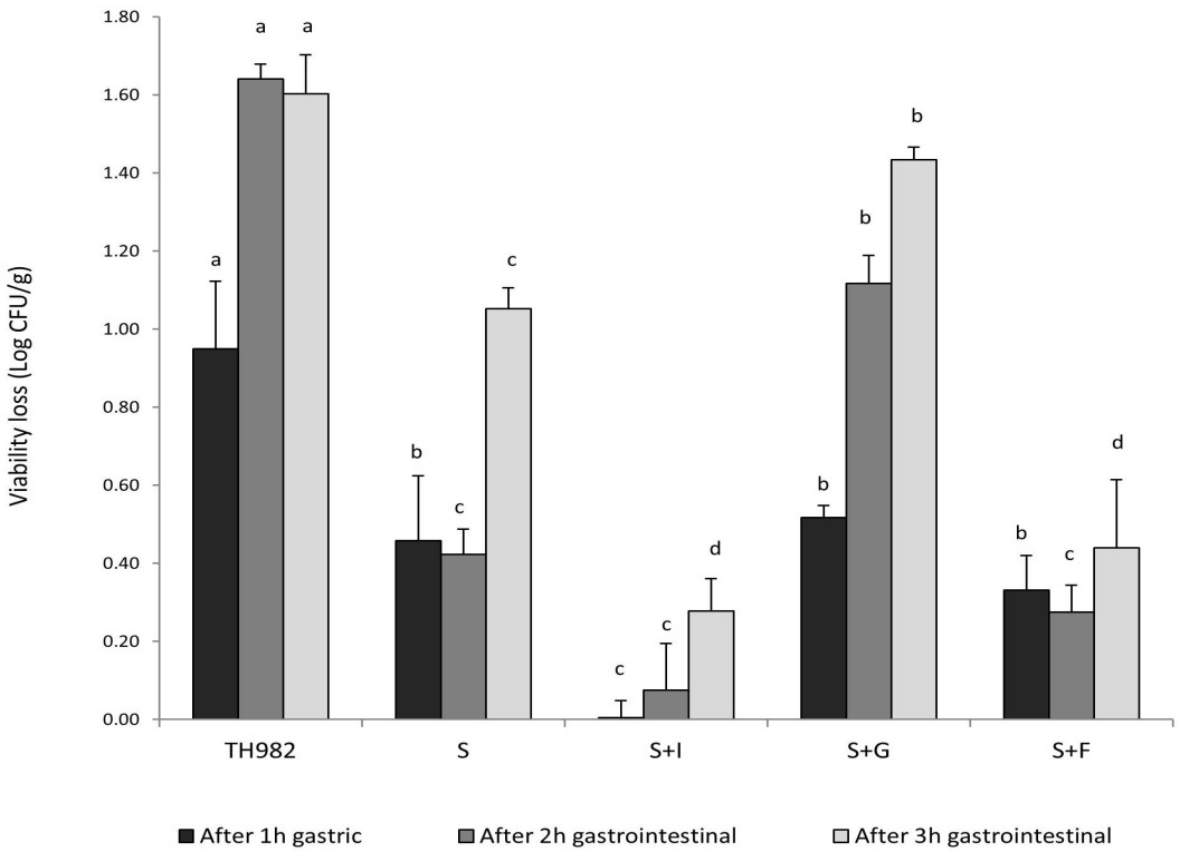

Figure 3.3. Survivability of encapsulated and free *S. thermophilus* TH982 cells during exposure to simulated gastrointestinal conditions. Different letters indicate significant differences within the same incubation step ($p < 0.05$) among different encapsulating matrix types.

S + G capsules showed low survivability during gastric environment transit; indeed, the reduction of viable cells was higher (0.52 log CFU/g decrease) than encapsulated cells with S + I (no reduction detected) after 1 h of gastric condition (pH 2.5). This fact might be due to a variation of pH, which changes the gelatin charge of amino and carboxyl groups so that the modification of cross-links and structure of the chain could influence the swelling behavior of gelatin capsules (153)

On the other side, an interesting outcome was given by the two types of prebiotics used as a co-encapsulating agent with sodium alginate. After 3 h of exposure to gastrointestinal conditions, S + I and S + F revealed the highest cell survivability without any significant difference, leading to a low cell reduction (less than 0.45 log CFU/g decrease for both agents). Prebiotics,

such as 2′-FL and inulin, which could provide good protection and even promote cell proliferation, appeared to contribute to the growth of *S. thermophilus* TH982. In addition, oligosaccharides are difficult to decompose by enzymes in digestive fluid but can be metabolized by beneficial bacteria in the colon (144). It has been further demonstrated that oligosaccharides contained in human milk have an extraordinary resistance to hydrolysis by digestive enzymes of the small intestine(154) .The combination of calcium alginate with prebiotics such as inulin improves the viability of probiotics and facilitates the formation of an integrated structure of capsules. Researchers found better protection of *Lactobacillus casei* and *Bifidobacterium bifidum* cells in coated capsules with prebiotics such as inulin after the gastrointestinal transit. The addition of prebiotics could be synergistic in gelling, and as a result, may help to maintain and improve the degree of protection of the bacterial cells (155).

Lastly, the free cell suffered more from the gastric conditions than that during the 3-h gastrointestinal transition. In contrast, encapsulated bacteria evidenced greater reductions during gastrointestinal transit than under gastric conditions alone. This behavior may be due to the fact that alginate is stable in low-pH solutions: the ionotropic alginate gel formed by Ca^{2+} cross-linking of carboxylate groups is insoluble at low pH, but exposure to neutral pH or higher solubilize the alginate, causing swelling (156) .

3.3.4. Release kinetics

The release of encapsulated *S. thermophilus* TH982 after incubation in simulated intestinal fluid was evaluated after 30, 60, 90, 120, 150 and 180 min, as reported in Figure 3.4. After 30 min of agitation at 100 rpm at 37 °C in 50 mM K_2HPO_4, a great release of cells encapsulated with S + F was detected. Considering the initial number of cells entrapped in this type of capsules (9.73 ± 0.04 log CFU/g of capsules), the number of cells released in the simulated intestinal fluid after 30 min was 8.83 ± 0.04 log CFU/mL of K_2HPO_4, representing a very high value compared with the other types of encapsulating matrices after 180 min, which resulted about 2-log lower. This result confirms what was visible during the experiment: after 30 min, there was an evident solubilization of S + F capsules, a characteristic not displayed by the others (S, S + I, and S + G). Moreover, the number of

released cells suggests that once liberated, *S. thermophilus* TH982 can withstand well simulated intestinal conditions, since after 180 min the number of cells released from S + F capsules was 9.72 ± 0.05 log CFU/mL, which means 99.89% of the initial entrapped cells.

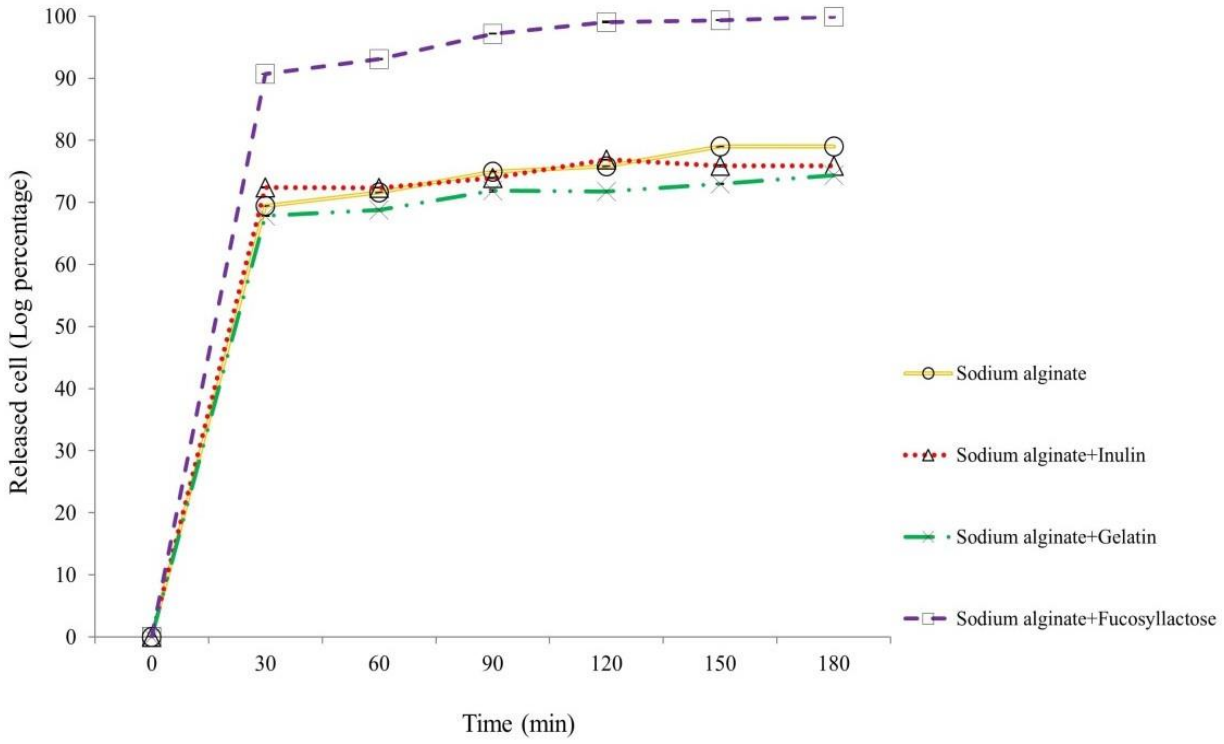

Figure 3.4. Release kinetics of entrapped *S. thermophilus* TH982 cells from different capsule formulations during incubation in a simulated intestinal environment.

It is essential to ensure that the capsules give protection through the simulated gastrointestinal passage and ensure that the encapsulation matrix allows a release of viable and metabolically active cells into the intestine (144, 157). The release of the cells from microcapsules in the colon is essential for the growth and possible colonization of probiotics (144, 158). In the absence of this property, the entrapped organisms and the capsules will be washed out from the body without exerting a significant beneficial effect (159). Many studies investigated the release of encapsulated probiotics in simulated intestinal fluid; however, to the best of our knowledge, there is no similar outcome from any encapsulating agent reported in the literature to date (139, 144, 157). Sabikhi et al. (159) reported the release of encapsulated *L. acidophilus* in alginate-

starch microspheres using a simulated colonic fluid with the same formulation. They found that the cell count after 150 min of incubation was 7.45 log CFU/mL, suggesting that all the microencapsulated cells were released at that time (initial number of 7.47 log CFU/g of capsule). Although 150 min is about the time needed for intestinal transit of microcapsules, they did not find a significant release after 30 min (159). In another study, a fully released *L. plantarum* encapsulated with sodium alginate and sodium alginate with inulin in a 60-min exposure to simulated intestinal fluid was reported. From the results, it was concluded that the release mechanism was probably due to the replacing of calcium ions in the encapsulation matrices. Also, capsules with inulin showed a faster release rate during the first 20 min, which could have been induced by the addition of inulin in sodium alginate affecting the binding of calcium ion (147).

A possible mechanism that could be involved in the fast release of *S. thermophilus* TH982 cells encapsulated with sodium alginate and 2′-fucosyllactose could be that this small soluble milk glycan used as a co-encapsulating agent in this experiment is formed by fucose linked to the two positions of β-Gal residues of lactose; hence no electrostatic repulsions may occur with the negatively charged carboxyl groups of sodium alginate structure. Therefore, the complete release of the cells is probably due to the vigorous interaction of the prebiotic structure with the divalent cations (Ca^{2+}) of the sodium alginate network, resulting in disintegration of the "egg-box" structure in K_2HPO_4. This process is well known and recognized to explain the inulin capsules releasing behavior in simulated intestinal fluid, a prebiotic commonly used as encapsulating material (147).

3.4. Conclusion

This study represents the first report among the available literature on the use of 2'-fucosyllactose, a trisaccharide from human milk, used as a prebiotic agent in microencapsulation of *S. thermophilus* cells. The alginate-based microcapsules showed interesting features, including good capability to protect bacterial cells from harsh simulated gastrointestinal conditions. Compared to other molecules used in microencapsulation together with alginate, such as gelatin and inulin, 2'-fucosyllactose evidenced an extremely quick (30 min) and abundant release of

bacterial cells from the capsules inside a simulated intestinal fluid. These results encourage to devise further studies aimed at testing these properties under in *vivo* conditions.

Chapter 4

Exopolysaccharide producing *Limosilactobacillus fermentum* ING8, a potential multifunctional starter strain with relevant technological properties and antimicrobial activity

Abstract

Lactic acid bacteria (LAB) have gained particular attention among different exopolysaccharide-producing microorganisms due to their safety status and effects on human health and food production. Exopolysaccharide-producing LAB play a crucial role in different ways, such as improving texture, mouthfeel, controlling viscosity, and for low-calorie food production. In this study, we isolated a multifunctional strain with good exopolysaccharide production properties. *Limosilactobacillus fermentum* ING8 was isolated from an Indian traditional fermented milk and evaluated for its safety, enzymatic activity, NaCl resistance and temperature tolerance, milk coagulation and storage stability. Finally, the complete genome of this strain has been sequenced and subjected to safety *in silico* evaluation and genomic analysis. The results reveal that *L. fermentum* ING8 possesses relevant technological properties, such as exopolysaccharide production, antimicrobial activity, and galactose utilization. Besides, this strain has shown very high stability to storage conditions at refrigeration temperature. In addition, the genomic analysis did not evidence any possible deleterious elements, such as acquired antibiotic resistance genes, virulence genes, or hemolytic-related genes. However, all structural genes related to the galactose operon and EPS production have been detected. Therefore, *L. fermentum* ING8 can be considered a promising multifunctional bacterium to be proposed as co-starter in different types of dairy productions.

4.1. Introduction

"*Dahi*", that means curd, is an Indian traditional fermented milk prepared from either cow, buffalo or goat milk, and it is considered one of the oldest consumed dairy products in India, Pakistan, Nepal, and Bangladesh (160). Due to the unknown composition of microbial starter cultures and the uncontrolled fermentation procedures, traditional fermented foods can have wide variability in their microbiological, nutritional, and functional properties (161). The *Dahi* made from buffalo milk is usually more acidic and contains a higher amount of protein, calcium, fat, and lactose, providing more energy than that made from cow milk (162). In addition, it is enriched with iron, phosphorus, vitamin A and natural antioxidants, while the cholesterol content is comparatively lower than cow milk (163). *Limosilactobacillus fermentum* is a Lactic Acid Bacterium (LAB), Gram-positive, non-spore forming, rod-shaped that produces organic acid

from the fermentation of carbohydrates (164). *L. fermentum* can also inhibit the growth of food-borne pathogens in food products (165) and it possesses the "generally recognized as safe" (GRAS), (166). Foods obtained from fermentation by *L. fermentum* usually possess palatability, high sensory quality, texture, stability and nutritional properties (167).

The main technological aspect which defines LAB is their capability to produce lactic acid by fermenting different sugars (168, 169). Among carbohydrates, galactose accumulation in dairy products following lactose fermentation, is problematic and challenging. The accumulation of galactose can cause a browning effect on heat-treated cheeses such as Asiago, Parmigiano Reggiano, and Grana Padano in different foods and can damage their appearance (170, 171). In addition, galactose accumulation in dairy products can lead to toxic effects on people suffering from galactosemia (172). Therefore, the use of starter cultures capable of utilizing galactose could be advantageous for the dairy industry. In addition to the production of lactic acid, LAB are used in the food industry for other relevant properties, such as their proteolytic and lipolytic activity, ability to synthesize a wide range of compounds such as organic acids, peptides, antimicrobial agents, aromatic compounds by the metabolism of citrate, and production of exopolysaccharides (EPS) (173). EPS play a crucial role in food production by improving texture in low-calories food products and in dietary fibers products (174, 175). In addition, they can be useful to the human health by providing beneficial effects such as cholesterol-lowering activity and immune-stimulating properties (176).

In this study, *Dahi* samples made from buffalo milk were collected from different household markets in India. The safety and technological properties of the newly isolated LAB strains were investigated by phenotypical approaches. Finally, the complete genome of the promising strain *L. fermentum* ING8 as a strong lactose/galactose utilizer with good EPS production was sequenced for further *in silico* evaluation and genomic analysis.

4.2. Material and Methods

4.2.1. Sample collection and isolation of new LAB strains

Dahi samples were collected from different villages of the Gadwal District Telangana state (India) during summer 2018. All samples were trasferred into sterile plastic tubes and stored at 4°C. For LAB strains isolation of LAB, 10 g of each sample were homogenized with 90 mL of sterile phosphate-buffered saline (PBS; NaCl 8.0 g/L, KCl 0.2 g/L, Na_2HPO4 1.44 g/L, KH_2PO4

0.24 g/L, pH 7.4). Then, decimal dilutions of the solution was prepared to up to 10^{-9} and all were inoculated on MRS (De Man Rogosa Sharp, Sigma-Aldrich, Saint Louis, MO, USA) agar using the pour plate method. Plates were incubated in anaerobic jars at 30°C for 72 h. Finally, one representative colony was taken from each sample, unless different colony morphology were detected. All the isolates were examined for preliminary phenotypical tests, i.e. Gram staining, microscopic morphology, catalase and oxidase. The stock cultures were prepared in MRS broth inoculated with 25% (v/v) glycerol and kept at -80 °C until further use.

4.2.2. DNA extraction and RAPD-PCR analysis

DNA extraction was done using a bacterial lysate protocol according to Tarrah *et al.* (55) with slight modifications. Briefly, 5 mL of overnight bacterial cultures were washed 2 times with sterilized PBS and added to 50 µl of lysis buffer (NaOH 0.05 M + SDS 0.25%). This solution was vortexed for 2 min and then incubated at 94 °C for 15 min in a thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). Finally, the supernatant was collected by centrifugation at 13,000g for 10 min and diluted at 1:100 in ultrapure sterile water. The RAPD-PCR analysis was performed using primer M13 (5' GAGGGTGGCGGTTCT 3') according to the protocol by Andrighetto *et al*. (56). RAPD-PCR profiles were analyzed using the software package Gel Compare Version 4.1 (Applied Maths, Kortrijk, Belgium) based on the Pearson product-moment correlation coefficient.

4.2.3. Lactose/galactose utilizers selection

Ten mL of overnight bacterial cultures were centrifuged at 5,000 rpm for 10min; the pellets were washed twice using 5 mL of PBS and resuspended in 5 ml of the same solution. Lactose and galactose (Sigma-Aldrich) solutions (10% w/v) were prepared separately, filtered using 0.22 µm filters and added to MRS glucose-free medium to a final concentration of 1%. Finally, the media (MRS+1% lactose/ MRS+1% galactose) were inoculated with 10^6 CFU/mL of overnight bacterial cultures and incubated at 37 °C for 24 h using 96-well microtiter plates (sigma SIAL0596, MO, USA) and a Tecan incubator/reader (spark 10M, Tecan GmbH, Grödig. Austria).

The optical density was measured for both lactose and galactose by reading the absorbance at 600 nm (OD600) every 30 min for 24 h. Blank and negative controls were inserted as well. The experiment was done using two biological and four technical replicates.

4.2.4. Exopolysaccharide production and quantification

Overnight bacterial cultures were streaked on a modified MRS agar medium containing 10% sucrose and incubated at 30°C for 3 days to allow EPS production. Strains generating slimy colonies were considered EPS producers (177). EPS producer strains on MRS agar were selected for EPS quantification using MRS broth containing 10% sucrose. An overnight culture (1%) was used to inoculate MRS broth containing 10% sucrose and incubated at 30°C for 3 days. Then the supernatant was collected by centrifugation at 5500 rpm for 20 min, and EPS was precipitated by using five volumes of cold 96% ethanol (Sigma-Aldrich) and incubating for 24 h. Finally, the EPS were collected by centrifugation at 4°C (5500 rpm for 20 min), dried at 60°C overnight, and the weight recorded (178). The experiment was done using two biological and three technical replicates.

4.2.5. Antimicrobial activity determination

Ten µL of 24 h cultures were placed on two MRS agar plates and incubated overnight. After the incubation, in one of the plates, 2 µL of proteinase K solution (20 mg/mL, pH 8) were spotted close to the bacterial growth to determine the production of bacteriocin compounds and incubated for 1h. Successively, a lawn of 4 mL of BHI soft agar (Sigma-Aldrich) containing 400 µL of an overnight culture of indicator strains, namely *Listeria monocytogenes* ATCC 19117, *Bacillus cereus* ATCC 11778, *Escherichia coli* APEC 18042/2 was poured on the top of the existing solid medium and plates were further incubated at 37°C for 24 h. After incubation, plates were inspected for zones of indicator strains growth inhibition surrounding the colonies. The presence of inhibition haloes in the MRS plate and the absence of haloes for the same strain in the protease-containing plate indicate the proteinaceous origin of the antimicrobial compound (64).

4.2.6. Safety assessments of *L. fermentum* ING8

4.2.6.1. Minimum inhibitory concentration (MIC)

The MIC test was performed in 96-well microtiter plates according to the broth microdilution method proposed by Wiegand et al. (61). The following antibiotics were chosen, according to the European Food Safety Authority (EFSA) recommendation: vancomycin, kanamycin, ampicillin, tetracycline, erythromycin, chloramphenicol, streptomycin, gentamicin and ciprofloxacin. The antibiotics were dissolved in MRS plus ISO-Sensitest (Sigma-Aldrich) broth in 1:9 ratio, serially diluted in 96-microtiter plates and further inoculated with bacterial cells (5×105 CFU/mL). The test was performed in 3 replicates, and the MIC value was considered the concentration present in the first well with no visible growth.

4.2.6.2. Hemolytic activity test

Twenty µL of overnight cultures were spotted on MRS plates containing 5% (w/v) sheep blood (Thermo Fisher Scientific, United States) and incubated at 37 °C for 48 h. Hemolytic activity was determined by a clear halo surrounding the colonies (62). *Staphylococcus aureus* ATCC 6538 and *Lactobacillus rhamnosus* GG were included as positive and negative controls, respectively.

4.2.7. Technological characterization of *L. fermentum* ING8

4.2.7.1. Enzymatic activities

The amylolytic activity was determined using a two-step procedure by first culturing the strain ING8 in a modified MRS broth (MRS with 0.25% starch instead of glucose), incubated at 37 °C for 24 h. Subsequently, 10 µl of the cultured bacteria were spotted on a medium containing 0.5% meat peptone, 0.7% yeast extract, 0.2% NaCl, 2% starch and 1.5% agar (Sigma-Aldrich) on plates incubated anaerobically at 37 °C for 48 h. The amylolytic activity was determined by detecting clear zones around the colonies after staining with Lugol solution.

The lipolytic activity was evaluated using a two-step procedure by culturing the bacteria in a modified MRS medium containing 1% olive oil and 1% Arabic gum, and incubating at 37 °C for 24 h. Successively, 10 µl of subcultured bacteria were spotted on a medium containing 0.1% tryptone, 0.5% yeast extract, 0.05% NaCl, 0.1, 0.5, or 1% olive oil, 1% Arabic gum, and 1.5%

agar (Sigma-Aldrich) and the plates were incubated anaerobically at 37 °C for 48 h. The lipolytic activity was evaluated by the detection of a clear zone around the colonies.

Regarding the proteolytic activity, the bacterial strain was cultured in MRS broth incubated anaerobically at 37 °C for 24 h. After incubation, 30µL of the supernatant were collected and spotted on a sterile paper disc, later placed over a medium containing 1% skim milk and 1.5% agar. Then plates were incubated anaerobically for 48 h at 37 °C. The clear zone around each disc was measured with a caliper to determine the level of proteolytic activity (179).

4.2.7.2. NaCl tolerance test

Strain ING8 was subcultured in MRS medium incubated at 37 °C for 24 h. After centrifugation, the pellet was washed two times with PBS and transferred to MRS supplemented with 2, 6 and 10% (w/v) NaCl, and incubated at 37 °C for 24 h. The growth ability was checked by reading the optical density (OD600nm) using 96-well microtiter plates (Sigma-Aldrich) and Tecan reader (Tecan-Magellan) (177). The experiment was done using two biological and three technical replicates.

4.2.7.3. Growth at different temperatures

The ability of bacterial growth at different temperatures was done using the protocol published by Ribeiro *et al*. with slight modification (177). Strain ING8 was cultured in MRS broth medium incubated at 30 °C, 37 °C, 45 °C, and 50 °C respectively for a period of 24 h. The growth was checked by reading the optical density (OD600nm) using 96-well microtiter plates (Sigma-Aldrich) and a Tecan reader (Tecan-Magellan). The experiment was done using two biological and three technical replicates.

4.2.7.4. Acidification and milk coagulation

The acidification and milk coagulation capability of strain ING8 were evaluated using a modified protocol by Shangpliang *et al*. (160). Briefly, the overnight culture of ING8 was centrifuged, the pellet washed two times using sterile PBS and resuspended in 5 mL of the same buffer. Later, 100 µL of this solution were used to inoculate 10 mL sterile of skimmed milk (10%) incubated at 30 °C. The pH value and coagulation status were determined after 3, 6 and 24 h. The experiment was done using two biological and three technical replicates.

4.2.7.5. Viability during storage

An overnight culture of ING8 was centrifuged, the pellet washed two times using sterile PBS, resuspended in 10 mL sterile skimmed milk (10%) and stored at 4 °C for 0, 7, 14, and 21 days (180). At each time interval, an aliquot was taken, serially diluted, and plated on MRS agar to enumerate viable bacteria. The experiment was done using three technical replicates.

4.2.8. Genome sequencing, assembly, and annotation of *L. fermentum* ING8

According to the manufacturer's instructions, genomic DNA from strain ING8 was extracted using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). The quality of the extracted DNA was checked spectrophotometrically (Spark 10M, Tecan). The genome sequencing was done using the paired-end sequencing technology with an Illumina MiSeq sequencer (Ramaciotti Centre for Genomics, Sydney, Australia). A Unicycler assembler was used to assemble the raw reads by using the PATRIC database server 3.6.12, setting the parameters on default (65).

The prediction and annotation of the assembled genome were carried out using the Rapid Annotation using Subsystems Technology (RAST) (66), and the graphical genome map of *L. fermentum* ING8 was constructed using *the* CGView (Circular Genome Viewer) (181) after scaffolding the assembled contigs using the medusa webserver (67) and the type strain *L. fermentum strain* DSM 20052 as the reference genome.

Genome stability and safety were assessed by studying the presence of virulence genes, plasmids and acquired antibiotic resistance genes within the genome of *L. fermentum* ING8 by using IslandViewer4, PlasmidFinder 2.0, and ResFinder 3.2 servers (71, 182, 183).

4.2.9. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using GraphPad Prism software (version 7, GraphPad Software, Inc., San Diego, CA).

4.3. Results and discussion

4.3.1. Strains isolation and RAPD-PCR analysis

Isolates from ten different colonies (ING1, ING2, ING3A, ING3B, ING4, ING5, ING6, ING7A, ING7B, ING8) were collected from MRS agar plates considering colony morphology. One

colony was taken from each plate unless different morphologies were observed on the same plate. All isolates were rod-shaped, Gram-positive, catalase-negative, and oxidase-negative. A RAPD-PCR analysis was performed to compare the isolates based on their electrophoretic profiles (Figure 1). According to the UPGMA analysis performed by Gel Compare software, isolates ING7B with ING8 and ING4 with ING5 showed highly similar profiles.

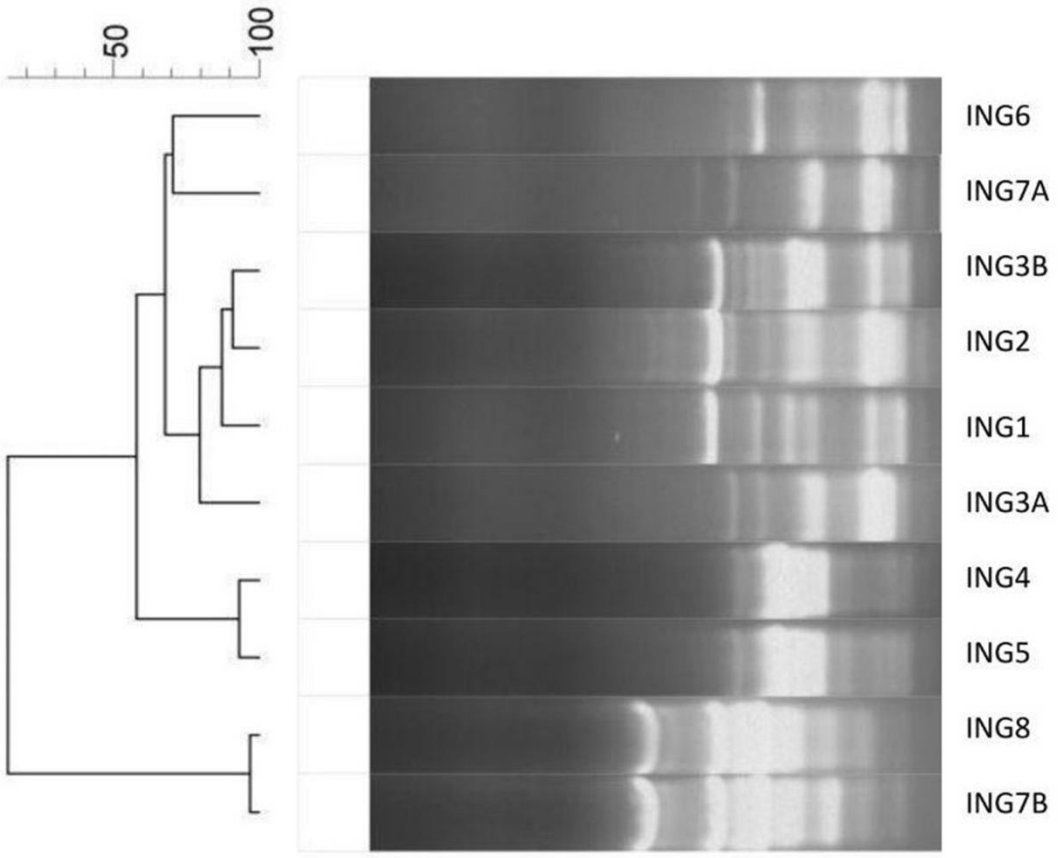

Figure 1. Cluster analysis of RAPD-PCR fingerprints of the isolates.

4.3.2. Lactose and galactose utilization

All tested strains could grow on a medium containing lactose, except strain ING4, which revealed a poor growth after incubation (OD600 = 0.2 was considered the growth threshold based on negative controls), whereas isolates ING7B and ING8 showed excellent growth

compared with the other strains (Figure 2-A). On the other side, all bacteria failed to grow on MRS medium containing galactose as the sole energy source except for ING7B and ING8 that revealed a very good capability to utilize galactose (Figure 2-B). Interestingly, isolates ING7B and ING8 overlapped entirely in galactose and lactose utilization dynamics, confirming the very high similarity detected by RAPD-PCR analysis, while ING4 and ING 5, that also showed very similar profiles, behaved very differently on lactose. For this reason, isolate ING7B was not considered for further studies. On the other side, carbohydrates, in general, are the primary energy source for fermentation and acidification by LAB. For the transport within the cell, different systems exist in bacteria. The main system is the PTS (Phospho Transferase System) but there is an alternative pathway for galactose. When galactose is imported into the cell by a specific permease, it uses the Leloir pathway to produce lactic acid as the final product. In galactose-positive strains, this sugar is metabolized by the Leloir pathway, which includes four different enzymes, namely galactose mutarotase (GalM), galactokinase (GalK), galactose-1-phosphate uridylyltransferase (GalT), and UDP-glucose 4-epimerase (GalE) (118). However, most LAB strains are galactose negative due to the weak induction of *gal* operon. Galactose accumulation in many kinds of cheese such as Asiago, Parmigiano Reggiano, Swiss, and Grana has been reported and resulted in discoloration and browning negative effects (170, 171).

Galactose accumulation can also be life-threatening due to the toxic effects on people suffering from galactosemia, a genetic disorder in humans (172). Therefore, galactose-positive strains can be useful for reducing such technological and health problems. In Parmigiano Reggiano and Grana Padano cheese production, 10–20% of *L. fermentum* is used as starter culture along with *Streptococcus thermophilus* and thermophilic lactobacilli (184). The availability of *L. fermentum* strains with a high level of galactose utilization and able to survive during prolonged storage periods, can be beneficial since the problems mentioned above can frequently happen in long ripened cheeses.

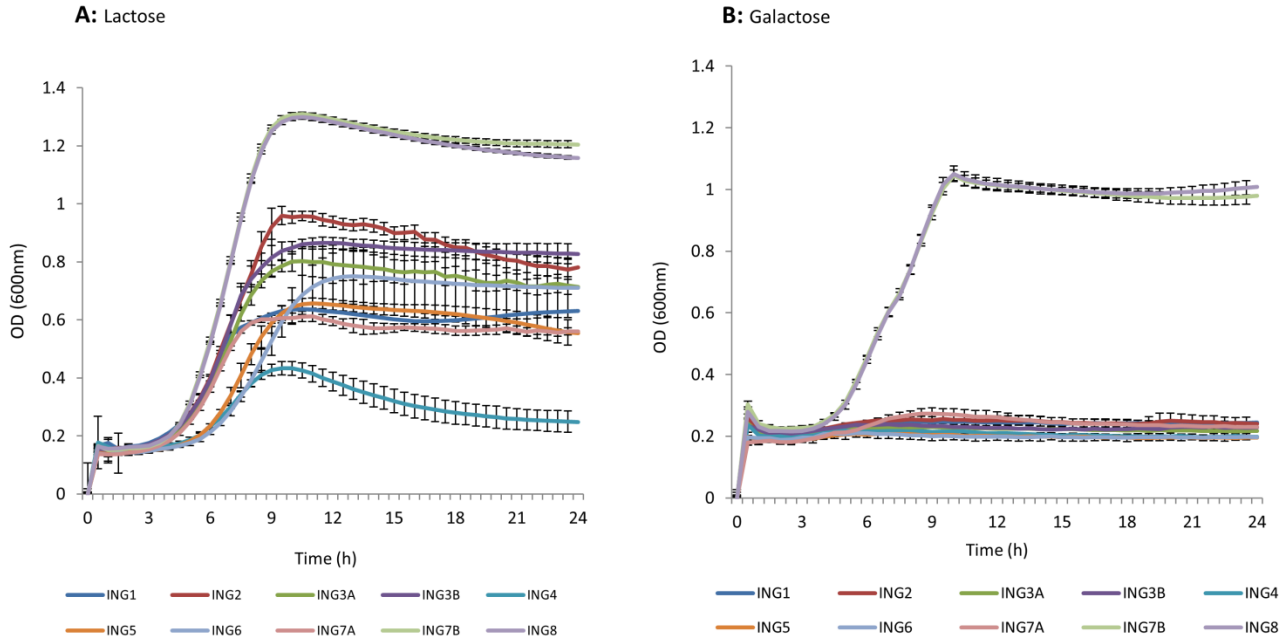

Figure 2. Growth curves of strains on lactose and galactose.

4.3.3. Exopolysaccharide production

Among all the strains examined for EPS production, ING8 was the only one producing sticky colonies (4 mm diameter) after 3 days at 30˚C using on MRS agar containing 10% sucrose. The quantification of EPS resulted in 400±18 mg/L. LAB have gained particular attention among different EPS producing microbes due to their application in the food industry and related health issues. This group of bacteria plays a crucial role in food production in different ways by controlling viscosity, improving texture, improving mouthfeel, freeze-thaw stability, being used in low-calorie food products, dietary fibers products, and so on (174, 175). On the other hand, LAB can be helpful to human health by providing some beneficial effects such as anti-cancer, anti-ulcer, antioxidant potential, cholesterol-lowering activity, and immune-stimulating properties (176). *L. fermentum* strains are used in various food products to increase their preservation, sensory characteristics, nutritional value, and other properties (167). This species is also naturally present in diverse cheeses due to its technological properties, such as exopolysaccharide production, to improve textural and organoleptic properties (167, 184). EPS producing bacteria can also reduce syneresis of fermented milks due to the water-holding properties of EPS, which can lead to a higher quality of low-fat dairy products (167). Many

studies have been conducted on the identification of EPS producing LAB from dairy environments due to its importance in food production (185). The amount of EPS produced by LAB is strain-dependent (185). In a study by Fukuda *et al.* (186), *L. fermentum* TDS030603 was found to produce a highly viscous EPS molecule with a yield of 100 mg/L. In another study reported by Wei *et al.* (187), the EPS amount produced by *L. fermentum* YL-11 was estimated at around 84.5 mg/L, and it indicated cytotoxic activity against HT-29 and Caco-2 colon related carcinogenic cells.

4.3.4. Antimicrobial activity determination

All the strains were tested for antimicrobial activity against food-borne pathogenic bacteria. Among all, only strain ING8 produced a growth inhibition zone greater than 31 mm against *Listeria monocytogenes* ATCC 19117, *Bacillus cereus* ATCC 11778, and *Escherichia coli* APEC 18042/2 (Table 1). Indeed, the same haloes were also detected in the plates added with proteinase K, indicating the non-proteinaceous nature of such inhibitory activity.

According to the results, strain ING8, due to its galactose utilization capability, antimicrobial activity, and high amount of EPS production was selected for further safety and technological assessments. Some LAB strains produce different antibacterial peptides named bacteriocins, which could inhibit other bacteria species, including pathogens (47). Indeed, *L. fermentum* ING8 did not show bacteriocinogenic activity against the food-borne pathogenic bacteria tested; however, it could contrast the growth of all indicator strains to a great extent, most probably because of organic acid production and pH lowering capability.

Table 1: Antimicrobial activity by using spot on lawn technique.

	ING 1	ING 2	ING 3A	ING 3B	ING 4	ING 5	ING 6	ING 7A	ING 8
L. monocytogenes ATCC19117	+	+	+++	+	++	+	+	+	++++
B. cereus ATCC 11778	++	+	+	+	+	+	+	+	++++
E. coli APEC18042/2	+	+	+	+	++	+++	+	+	++++

*Inhibition diagonal (mm) diameter≤12= (+); 13≤diameter≤20=(++); 21≤diameter≤30= (+++); diameter≥31= (++++).

4.3.5. Safety assessments of *L. fermentum* ING8

The MIC for nine antibiotics, recommended by the European Food Safety Authority (EFSA) was evaluated for strain ING8. As a result, the strain was susceptible to tetracycline, erythromycin, ampicillin, chloramphenicol, gentamycin, streptomycin, and ciprofloxacin. However, it was resistant to kanamycin and vancomycin (Table 2). On the other side, strain ING8 did not reveal any hemolytic activity when spotted on MRS plates containing 5% (w/v) sheep blood.

Table 2. MIC values for human and veterinary relevant antibiotics. Breakpoints (µg/mL) suggested by EFSA are reported in parentheses.

	Antibiotics								
Strain/Antibiotics	chloramphenicol	Gentamicin	tetracycline	erythromycin	ampicillin	streptomycin	vancomycin	Ciproflox acin	Kanamycin
ING8	4	8	<0.125	0.5	0.5	16	>64	4	32

* Strains with MIC higher than the breakpoint (in bold) are considered resistant.
**n.r. not required by EFSA.

4.3.6. Technological properties of *L. fermentum* ING8

Proteolytic, lipolytic and amylolytic activities assessments did not reveal the presence of any of these properties in strain ING8 (Table 3). Regarding NaCl tolerance, ING8 could grow well in MRS containing 2% and 6% NaCl but it was not able to grow in the presence of 10 % NaCl (Table 3). On the other hand, it showed optimal capability to grow at 30 ˚C and 37 ˚C, and very good to 45 ˚C and 50 ˚C, with relatively good acidification capability (around pH 4) after 24h (Figure 3-A) and coagulation ability (after 24h) tested in 10% sterile skimmed milk. Regarding the viability during 21 days of storage, we did not detect any reduction in viability of ING8 in 10% skimmed milk stored at 4˚C ($p \leq 0.05$) (Figure 3-B). Technological properties such as proteolytic, lipolytic, and amylolytic activities of LAB can positively affect texture and flavor of the products. In addition, starter cultures are usually exposed to some harsh conditions such as high temperatures or high NaCl concentration, depending on the type of cheese, which can cause early autolysis of starter cultures due to osmotic stress (188).

Table 3. Technological properties of EPS producer strain *L. fermentum* ING8.

	Different temperatures (C°)*				Different NaCl (%)**						
	30	37	45	50	0	2	6	10	Proteolytic ***	Lipolytic***	Amylolytic***
L. fermentum ING8	+++	+++	++	++	+++	+++	++	-	-	-	-

*Growth measured by spectrophotometric method: OD≤0.05 = (-); OD 0.05≤0.1= (+); OD≤0.5=(++); OD>0.5= (+++).
** Growth measured by spectrophotometric method: OD≤0.05 = (-); OD 0.05≤0.1= (+); OD≤0.5=(++); OD>0.5= (+++).
***positive result (+), negative result (-)

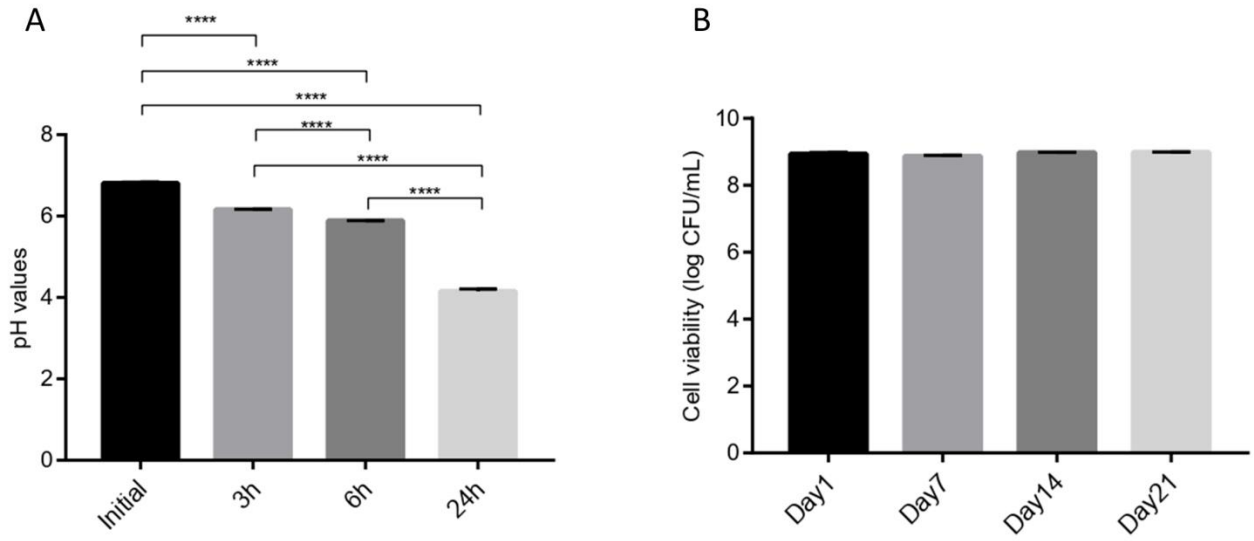

Figure 3. A: pH reduction and acidification of *L. fermentum* ING8, B: Survivability of *L. fermentum* ING8 cells during 21 days.

4.3.7. Genomic analysis of *L. fermentum* ING8

The assembled genome of the EPS producer *L. fermentum* ING8, generated *25 scaffolds (158 contigs), giving a full genome size of 1.98* Mb with 51.3 GC% content (Table 4). The circular graphical genome map of *L. fermentum* ING8 is reported in Figure 4. The gene prediction done by RAST revealed a total of 2103 protein-coding sequences (CDSs) classified in 208 SEED subsystems. Fifty-eight structural RNAs and 13 genes related to osmotic, oxidative, and

detoxification stress responses were predicted after the annotation (Figure 5). Neither virulence-related genes nor acquired antibiotic resistance and plasmid sequences were detected after the annotation (Table 4). The largest section of the ING8 genome subsystem is dedicated to amino acids and derivatives (16.32%), followed by protein metabolisms (13.37%) and carbohydrate metabolism with 12.85%, respectively (Figure 5). Moreover, a deep search within the genome of *L. fermentum* ING8 revealed the presence of the complete gal operon with all its structural genes (*galK*, *galT*, *galE*, *galM*, *galR*, *and galA*) and of EPS production-related genes (*epsB*, *epsC*, *epsD*, and *epsE*). Finally, microbes that are used to produce food should be safe. They should come from the qualified list established by EFSA according to their taxonomy and a very long periods of safe use, and must be examined for the presence of acquired and transmissible antibiotic resistance genes within their genomes as well as hemolytic-related genes (62). Therefore, the genome sequencing of new strains has becomes mandatory to precisely determine their taxonomy and retrieve all safety-related information present inside the genome (36, 89).

Table 4. Genome features of *L. fermentum* ING8.

Feature	Value
Genome size	1,981,384
G+C content (%)	51.3
Contig N50	41442
Contig L50	17
Number of contigs	158
Number of Protein Coding Sequences (CDSs)	2103
Number of tRNAs	57
Number of rRNAs (5S, 16S, 23S)	3
Number of genes related to plasmid and acquired antibiotic resistant gene	0
Number of genes related to Virulence, Disease, and Defense	0

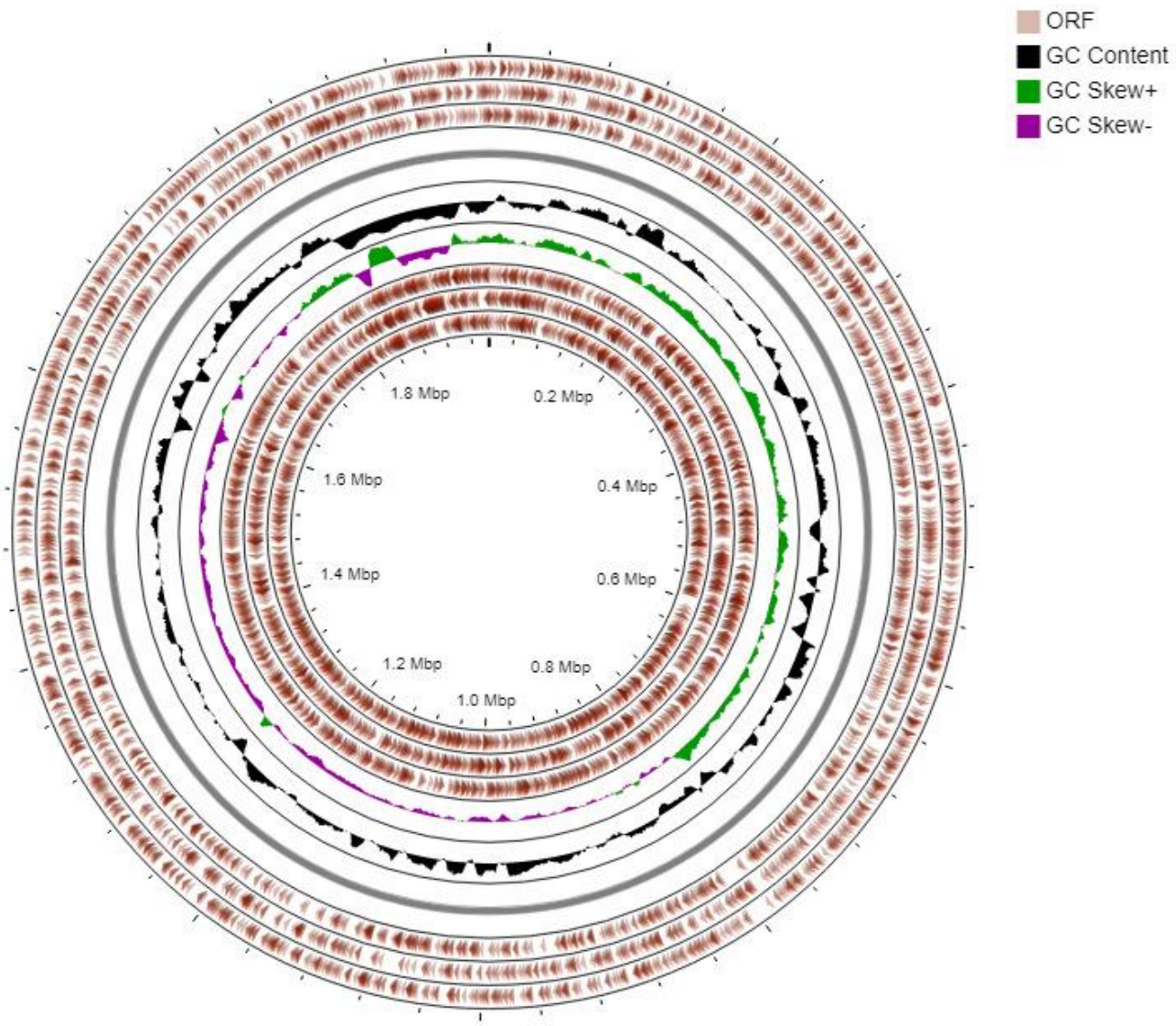

Figure 4. The circular graphical genome map of *L. fermentum* ING8, from outer to inner rings, ORF on the forward strand, GC content, GC skew, and ORF on the reverse strand.

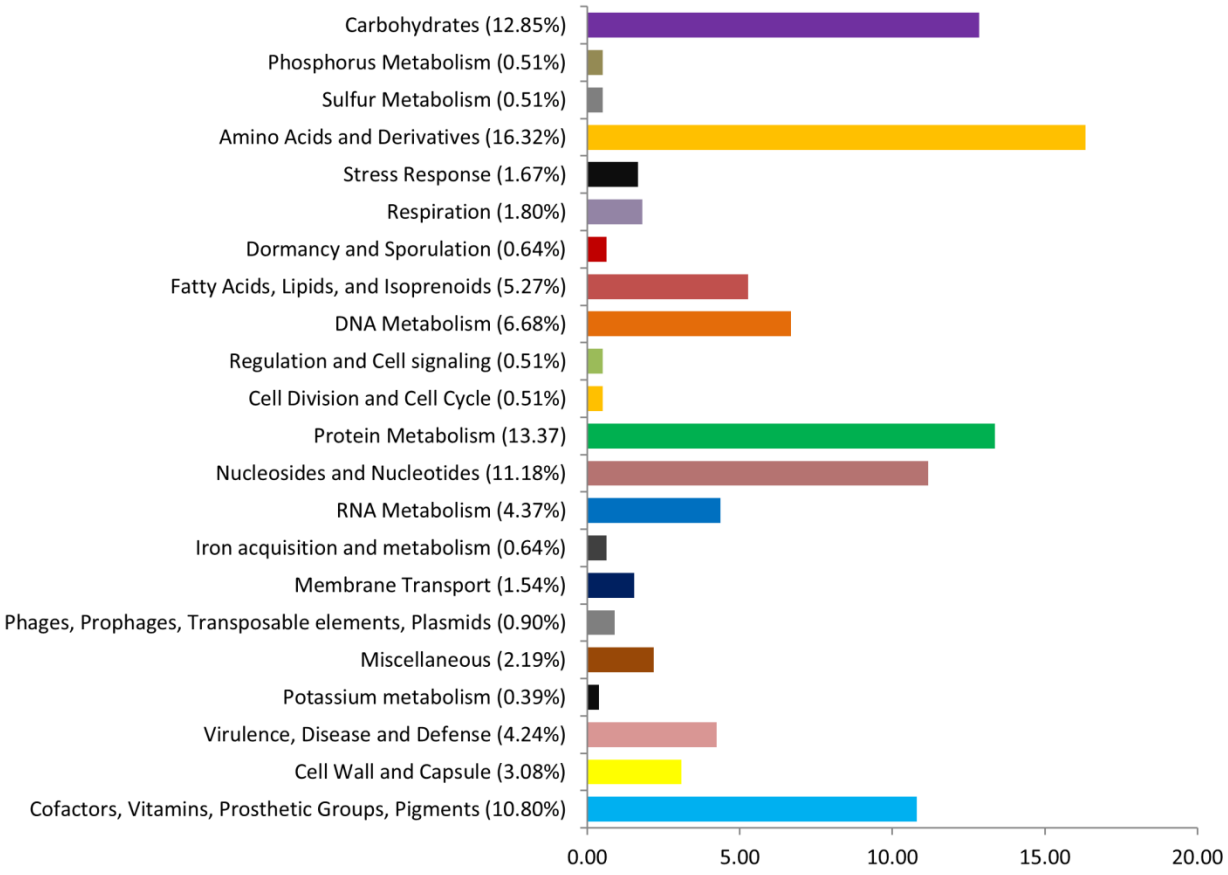

Figure 5. The overview of the RAST annotation and subsystems for *L. fermentum* ING8 genome.

4.5. Conclusion

In this study, we have isolated and studied *L. fermentum* ING8, a strain capable of galactose utilization with antimicrobial activity and good EPS production, which, interestingly, did not show any viability loss during 21 days of storage at refrigeration temperature. Besides, *L. fermentum* ING8 revealed relatively good tolerance to high temperatures and high concentrations of NaCl. On the other side, the deep genomic analysis of this strain did not reveal any possible deleterious characteristics such as presence of virulence genes, acquired antibiotic resistance genes or hemolytic-related traits. For these reasons, this strain has a good potential to be used as a multifunctional co-starter culture to produce different dairy products.

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