

Article

Usefulness of Potentially Probiotic *L. lactis* Isolates from Polish Fermented Cow Milk for the Production of Cottage Cheese

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Abstract: *Lactococcus lactis* bacteria are used as starter cultures in cottage cheese and fermented milk production due to their acidification and contribution in the creation of the characteristic sensory features. The aim of the research was to carry out isolation, genetic identification, and verification of the probiotic properties of selected *Lactococcus lactis* isolates obtained from Polish fermented cow milk and apply the best strains to produce cottage cheese with good rheological and sensory characteristics. The isolates obtained were identified morphologically, biochemically, and with the use of 16S rRNA gene sequencing. After pre-screening two of the tested *Lactococcus lactis* strains, A13 and A14 were observed to be most tolerant to high NaCl concentrations and bile salts and to acidify milk the most. We confirmed the activity of A13 and A14 against such pathogenic strains as *Escherichia coli*, *Salmonella enterica* ssp. *enterica*, *Salmonella enterica* ssp. *enterica* sv. *anatum*, *Staphylococcus aureus*, and *Enterococcus faecalis*. As a potential industrial starter culture with probiotic potential, the selected *Lactococcus lactis* A13 and A14 strains produced cottage cheese quickly with good sensory (colour, smell, taste, texture) and rheological (viscosity, elasticity) properties.

Keywords: *Lactococcus lactis*; milk clot; cottage cheese; screening



Citation: Pytka, M.; Kordowska-Wiater, M.; Wajs, J.; Glibowski, P.; Sajnaga, E. Usefulness of Potentially Probiotic *L. lactis* Isolates from Polish Fermented Cow Milk for the Production of Cottage Cheese. *Appl. Sci.* **2022**, *12*, 12088. <https://doi.org/10.3390/app122312088>

Academic Editor: Ioannis G. Roussis

Received: 28 October 2022

Accepted: 23 November 2022

Published: 25 November 2022

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1. Introduction

Lactococcus lactis strains are widely used as starter bacteria in the production of cottage cheese and fermented milk. They not only produce lactic acid, but also contribute to the sensory features of cheese. They belong to the order *Eubacteriales* and the family *Streptococcaceae*. *L. lactis* are Gram-positive cocci forming single pairs or short chains (0.5–1.5 µm) in their growth medium. These facultative anaerobic and catalase-negative bacteria do not form spores. The optimal temperature for their growth is 30 °C, but they can also grow between 10 °C and 37 °C at higher salt concentrations (>4% sodium chloride) [1]. The production of acid from different sugars by these microorganisms varies depending on the species. They are not able to grow in a medium containing 6.5% NaCl or at pH 9.6. They play important roles in the protection and safety of dairy products through the secretion of antimicrobial agents, e.g., lactic acid, diacetyl, hydrogen peroxide, and bacteriocins. In addition to the morphological and biochemical properties of *Lactococcus* strains, it is important to determine the technological and probiotic potential of strains isolated from fermented milk [2,3].

Lactic acid bacteria (LAB) present in raw milk mainly come from teats and udders, milking and storage equipment, and the animal's environment [4]. Therefore, wildtype lactococci isolated from milk can be new strains with desirable properties [5]. For easier and more effective isolation of strains, raw milk should be subjected to spontaneous lactic fermentation, which contributes to the multiplication of bacteria; interruption of the process in a timely manner helps to isolate lactococci. Their ability to ferment lactose is critically

important, particularly for their use as starter cultures in milk processing [6,7]. In cheese production, lactococci not only ferment lactose, lower the pH and cause milk to coagulate, but they also take part in the formation of specific sensory characteristics based on the enzymatic breakdown of milk proteins and fats [8]. Scientists and practitioners believe that the selection of autochthonous strains adapted to production conditions, is a guaranteed method of reproducibility and standardisation of the product without losing its typical features [9].

Despite the availability of various types of starter cultures containing *L. lactis*, there is still a need to look for new strains combining the desired technological features (resistance to various NaCl concentrations or temperatures and acidifying abilities) and primarily probiotic functional features, taking into account safety aspects. Probiotic microorganisms should survive in the human digestive tract and help in the maintenance of intestinal microbiota. They should also be resistant to antibiotics and exert health-promoting effects such as immunomodulation, anti-inflammatory activity, antagonistic activity against pathogens, inhibition of toxin activity, enhancement of nutrient bioavailability, and reduction in lactose intolerance [10,11]. The safety of probiotic strains for the consumer is also very important, and this includes e.g., non-pathogenicity and non-toxicity, appropriate origin, and lack of haemolytic potential and gelatinase activity. Among the features related to non-toxicity, the lack of amino acid decarboxylases is important, because biogenic amines cause a number of adverse effects in consumers, e.g., headaches or skin irritation [12]. These properties should be confirmed by the GRAS (generally regarded as safe) status [13]. Strains intended for starter cultures should also be viable during the technological processes and storage of products. Therefore, the use of a good starter culture containing defined strains can guarantee the safety, quality, and acceptability of fermented dairy products obtained in a traditional and innovative way [14].

The aim of the research was to carry out the isolation, genetic identification, and verification of the probiotic potential of selected *Lactococcus lactis* strains isolated from Polish fermented cow milk. The ability of the best strains to produce cottage cheese with good rheological and sensory characteristics was tested.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

Lactococcus lactis isolates were deposited in the laboratory collection of the Department of Biotechnology, Microbiology, and Human Nutrition, University of Life Sciences in Lublin. *Lactocaseibacillus rhamnosus* GG was obtained from the probiotic preparation Dicoflor (Bayer, Warsaw, Poland). Starter S1: *Lactococcus lactis* subsp. *cremoris*, *Leuconostoc* sp., *Lactococcus lactis* subsp. *lactis*, and *Lactococcus lactis* subsp. *lactis* biovar *diacetilactis* (Chr Hansen, FD-DVS CHN-19) and S2: *Lactococcus lactis* subsp. *cremoris*, *Leuconostoc* sp., *Lactococcus lactis* subsp. *lactis*, and *Lactococcus lactis* subsp. *lactis* biovar *diacetilactis* (Chr Hansen, FD-DVS FLORA DANICA) were the commercial cultures used for the production of cottage cheese. *L. lactis* strains were cultivated in M17 broth (BTL, Łódź, Poland) containing 1% glucose (GM17) and were stored at -80°C in the same medium with 20% glycerol [15].

2.2. Screening for *Lactococcus lactis*

In total, 30 samples of milk from dairy cows from eastern Poland were screened for the presence of *Lactococcus lactis* strains. The samples were incubated at 30°C for 24 h. After fermentation, bacteria were isolated in M17 agar. After 48 h, colonies of typical LAB were selected [16]. Then, they were introduced in GM17 liquid medium, incubated, and characterized morphologically using Gram staining. The purple cocci were photographed using an Evolution 300 light microscope (Delta Optical, Warsaw, Poland). The ability to produce catalase was then tested with H_2O_2 . Moreover, the growth of the 26 selected *L. lactis* strains in GM17 medium was observed after cultivation at the temperatures of 10°C , 30°C , 37°C , and 45°C and 2%, 4%, and 6.5% NaCl concentrations [14].

2.3. DNA Extraction and PCR Amplification of the 16S rRNA Region

DNA was extracted from overnight-incubated pure cultures using Genomic Mini AX Bacteria+ Kit (A&A Biotechnology, Gdańsk, Poland) according to the manufacturer's instructions and was used directly for PCR amplification in a thermocycler T100 Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA). PCR was set up in a master mix containing 7.5 µL PCR mix, 0.38 µL deionized water, 0.5 µM of each primer (212f (5'-GATGCAATTGCATCACTCAAAG-3') and 1406r (5'-ACGGGCGGTGTGTRC-3')), and 5 µL of each DNA sample. The PCR reaction was performed as described by Zycka-Krzesinska et al. [17]. The amplified products were analysed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide and visualized using a UV transilluminator Gel-Doc™ XR+ (Bio-Rad Laboratories Inc., Hercules, CA, USA). The Nova 1 kb DNA ladder (Novazym) was used as a marker. Sequencing of the 16S rRNA region of 1194 bp was performed by Genomed (Warsaw, Poland). The sequence of the PCR product was compared to the known sequences of the 16S rRNA genes in the NCBI GenBank database by alignment with the BLAST program [18]. Sequence similarity values were calculated from the multiple alignment using the BioEdit Sequence Alignment Editor. The evolutionary analysis was conducted in MEGA11. The phylogenetic tree was created based on the 16S rRNA gene sequences downloaded from GenBank using the maximum likelihood method with the best-fit Kimura 2-parameter model with a discrete gamma distribution (+G). To evaluate the statistical support of the branches, 1000 bootstrap replication was applied. The GenBank accession numbers for the 16S rRNA gene sequences were OP811530 and OP811531 for the A13 and A14 strains, respectively.

2.4. Biochemical Characterisation

Carbon source assimilation profiles of the isolates were determined using commercial API 50CH strips (bio-Merieux, Craponne, France). The tests were performed according to the producer's instruction using fresh cultures of 26 isolates incubated in GM17 medium at 30 °C. After centrifugation (14,000 rpm, 10 min, 4 °C) cell biomass was resuspended in API 50 CHL medium to an optical density of 2 according to the McFarland scale. The inoculated strips were incubated at 37 °C for 24 h and the change in colour in the wells was observed [19].

The enzymatic activities of the 26 strains were determined with the semiquantitative API ZYM system (bio-Merieux, Craponne, France) following the manufacturer's recommendations. Cell suspensions of fresh cultures (5 in the McFarland standard) were transferred into the wells of the API ZYM strips and incubated for 4 h at 37 °C; then one drop of developing reagents ZYM A and ZYM B was added to each of the wells [20]. Observations of colour changes, indicating positive reactions were made, and the evaluation was made on the basis of the API ZYM colour chart.

2.5. Probiotic Features of *L. lactis* Strains

2.5.1. Acid and Bile Tolerance

Studies on the tolerance of LAB cultures to high concentrations of bile salts (Sigma-Aldrich, Warsaw, Poland) and low pH were performed as described by Bhushan et al. [21]. The two best isolates of *L. lactis*, A13 and A14, were taken from the MRS overnight cultures and *L. rhamnosus* GG was used as a control and they were resuspended in 3 mL of pH 7.2 phosphate buffer. Cell suspensions with a cell density of 10^9 cells/mL were tested in MRS medium with 0.3% and 1% bile salts or in MRS medium with pH 7 (control), pH 3, and pH 2 for 0, 2, and 3 h. After incubation at 37 °C, decimal dilutions of the samples were made and plated on MRS agar. After 24 h of incubation, bacterial colonies (CFU/mL) were counted [21].

2.5.2. Antimicrobial Activity

The antibacterial activity towards various pathogens was evaluated using the well-diffusion method according to Feng et al. [22] with slight modification. Indicator bacteria,

i.e., *Staphylococcus aureus*, *Salmonella enterica* ssp. *enterica* sv. *anatum*, *S. enterica* ssp. *enterica*, *Bacillus cereus*, *Listeria monocytogenes*, *Escherichia coli*, and *Enterococcus faecalis* (approx. 10^8 CFU/mL), in a volume of 100 μ L of overnight cultures were inoculated over the entire surface of the Petri dishes with nutrient agar (BTL, Łódź, Poland). Holes were cut in the inoculated plates using a corkborer with a diameter of 8 mm, and 24 h liquid cultures of the *Lactococcus* A13 and A14 strains were spotted into them. The plates were incubated at 37 °C for 48 h and clear inhibition zones around wells were measured [23]. Each strain was tested in triplicate.

2.5.3. Autoaggregation/Coaggregation

The autoaggregation and coaggregation assays were performed according to the procedure described by Polak-Berecka et al. [24]. Suspensions ($OD_{600} = 1.0$) of one-day cultures of the two *L. lactis* strains, A13 and A14, and pathogens: *S. aureus* and *S. enterica* ssp. *enterica* sv. *anatum* were prepared in PBS buffer (pH 7.2). The OD_{600} values were measured every hour for 5 h and after 24 h without disturbing the bacterial suspensions using a spectrophotometer Biophotometer D30 (Eppendorf AG, Hamburg, Germany). In the coaggregation assay, the suspensions of lactococci and pathogenic bacteria were mixed in equal volumes and the OD_{600} values were measured as described above to determine the sedimentation kinetics. The measured OD_{600} values were used to calculate the aggregation and coaggregation coefficients. The analysis was performed in triplicate.

Aggregation coefficient (AC_t):

$$AC_t = \left[1 - \frac{OD_t}{OD_0} \right] \times 100$$

Coaggregation coefficient (CC_t):

$$CC_t = \frac{[(OD_L + OD_P)/2] - OD_t(L + P)}{OD_L + OD_P/2} \times 100$$

where: OD_0 is the optical density at time 0; OD_t is the optical density at time t ; OD_L is the optical density for *L. lactis* at time 0; OD_P is the optical density for the pathogen at time 0; and $OD_t(L + P)$ is the optical density for the *Lactococcus* + pathogen mixture at time t .

2.6. Haemolytic Activity

The haemolytic activity of the A13 and A14 strains was analysed on Columbia Lab-AgarTM supplemented with 5% defibrinized sheep's blood (BioMaxima S.A., Lublin, Poland). Day-old liquid cultures in M17 were spotted (40 μ L) on plates and incubated at 30 °C for 24 h. Then, the haemolysis zones were observed. *S. aureus*, previously cultured in nutrient broth for 1 day, was the positive control strain for β -haemolysis.

2.7. Technological Features of *L. lactis*

2.7.1. Ability of the Selected Strains to Acidify Milk and Form a Clot

For this experiment, fresh cultures of A13 and A14 strains were used as suspensions in 0.85% (w/v) NaCl with a turbidity similar to the McFarland standard No. 1 ($\sim 3 \times 10^8$ CFU/mL) [25]. The acidifying ability of the *L. lactis* isolates was assessed by addition of 1% culture aliquots to pasteurized (70 °C, 30 min) skimmed milk (10% w/v , POLSERO), followed by an incubation at 30 °C for 24 h. During the incubation, pH measurements were carried out at time 0 and after 3, 6, 9, 12 and 24 h using a digital pH meter (Hanna Instruments, H1 221, Olsztyn, Poland).

2.7.2. Rheological Properties of Milk Clot

The rheological measurements of clot formation were conducted using a Kinexus pro+ rheometer (Malvern Instruments Ltd., Worcestershire, UK). *L. lactis* A13 and A14 cultures and a mixture of both A13 + A14 strains, as well as S1 and S1 starter cultures and mixtures of

A13 + S1, A13 + S2, A14 + S1, and A14 + S2 were used in the study. Temperature control was maintained by the Peltier system of the rheometer. All rheological data were collected and calculated by rSpace software version 1.70.2180 (Malvern Instruments Ltd., Worcestershire, UK). The rheological measurements were carried out using a concentric-cylinder-fixed cup and a rotating bob. In order to prevent evaporation, 7 mL of oil was poured onto the sample's surface. The measurements were conducted at a frequency of 0.01 Hz. The dynamic measurements were carried out at 30 °C for 21 h (the strain corresponding to the maximum found within the linear viscoelastic region of the examined sample) [26].

2.7.3. Organoleptic and Flavour Evaluation

Skimmed milk powder obtained from POLSERO was pasteurized at 70 °C for 30 min. A 1% inoculum of cultures A13, A14, S1, or S2 grown in pasteurised skimmed milk was added to 300 mL of sterile skimmed milk in beakers and incubated at 37 °C for 24 h. The samples obtained were poured into nylon bags and kept for 12 h in refrigerated conditions to drain the cheese. Then, the colour, smell, texture, moisture, and taste of the obtained cottage cheese were assessed. The tests were carried out in accordance with the PN-A-86300: 1991 standard by ten trained panellists [27]. The curd cheeses collected from the tests were each subjected to a sensory evaluation using a five-point scale.

2.8. Statistical Analysis

Data are expressed as mean $\pm$ standard deviation ($n = 3$). Differences among the mean values were tested at $p < 0.05$ using an ANOVA and Fisher's test (STATISTICA 13, StatSoft Inc., Tulsa, OK, USA).

3. Results and Discussion

3.1. Screening for *Lactococcus lactis*

After the screening procedure, 105 typical LAB colonies were isolated from the fermented milk samples. Only 26 strains that were catalase-negative and Gram-positive cocci which acidified the cow milk the fastest were selected for further research. All selected strains grew in M17 liquid medium with 1% glucose at 30 and 37 °C, and with addition of 2, 4 and 6.5% NaCl (Table 1). Only two isolates did not grow at 10 °C (7.7%). The best growth of all the strains was observed at 30 and 37 °C and in media supplemented with 2 and 4% of NaCl. Two isolates, A13 and A14, showed the best growth ability during the experiment even in the presence of 6.5% NaCl, which is a prerequisite for their potential use as starter cultures. A similar screening procedure was described by Karakas-Sen et al. [5], Kimoto-Nira [28], Rasovic [29], and Dahou [30], where the researchers obtained 29 and 14 isolates confirmed as *Lactococcus lactis* from milk, 40 isolates from dairy products, and 10 isolates from Algerian cheese "J'ben of Naama", respectively. In contrast to strains A13 and A14, raw milk isolate IBB109 and bryndza cheese isolate IBB417 showed no growth and a 5-fold reduction in growth in the presence of 5% NaCl, respectively [11].

Table 1. Growth of 26 selected strains at various temperatures and salt concentrations after 48 h of incubation.

Strain	Temperature				Salt		
	10 °C	30 °C	37 °C	45 °C	2%NaCl	4%NaCl	6.5%NaCl
A1	+	++	++	-	++	++	+
A2	+	++	++	-	++	++	+
A3	+	++	+	-	++	++	+
A4	+	++	++	-	++	++	+
A5	+	++	++	-	++	++	+
A6	+	++	++	-	++	++	+
A7	-	+	++	-	++	++	+
A8	+	++	+	-	++	++	+

Table 1. Cont.

Strain	Temperature				Salt		
	10 °C	30 °C	37 °C	45 °C	2%NaCl	4%NaCl	6.5%NaCl
A9	+	++	++	-	++	++	+
A10	+	++	++	-	++	++	+
A11	+	++	++	-	++	++	+
A12	+	++	++	-	++	++	+
A13	+	+++	+++	-	++	++	++
A14	+	+++	+++	-	++	++	++
A15	+	++	+	-	++	++	+
A16	+	++	++	-	++	++	+
A17	+	++	++	-	++	++	+
A18	+	++	++	-	++	++	+
A19	+	++	+	-	++	++	+
A20	+	++	++	-	++	++	+
A21	+	++	++	-	++	++	+
A22	+	++	++	-	++	++	+
A23	-	++	++	-	++	++	+
A24	+	++	+	-	++	++	+
A25	+	++	++	-	++	++	+
A26	+	++	++	-	++	++	+

+ weak growth, ++ good growth, +++ very good growth, - no growth.

3.2. Identification of *Lactococcus lactis* by Species-Specific PCR

Genetic identification of LAB bacteria isolated from milk or non-dairy raw materials by sequencing the 16S rRNA gene with the use of various universal primers is a popular and reliable technique practiced in recent years [29,31,32]. Exactly 26 LAB isolated from the fermented milk were subjected to *L. lactis*-specific PCR. A specific 1200 bp DNA amplification fragment was confirmed in all isolates (Figure 1). These PCR-positive *L. lactis* isolates were further tested by sequencing the 16S rRNA region. The 1089 bp long partial sequence of the 16SrRNA gene of the strains studied showed 99.8% sequence similarity to each other. These sequences displayed the highest similarity to the 16S rRNA gene sequences reported from *L. lactis* reference strains (99.7%). The phylogenetic analysis placed the A13 and A14 isolates in a cluster together with *L. lactis* strains (Figure 2), which confirmed that they belonged to the species *L. lactis*.

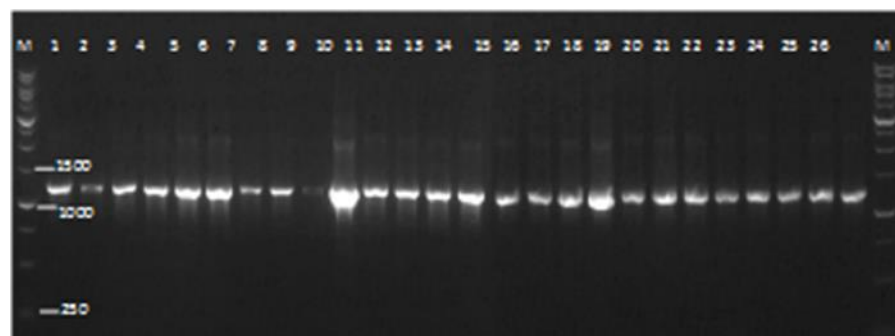


Figure 1. Electrophoretic separation of the PCR products of *L. lactis*. M—marker—Nova 1 kbp ladder (Novazym), 1–26 numbers of isolates.

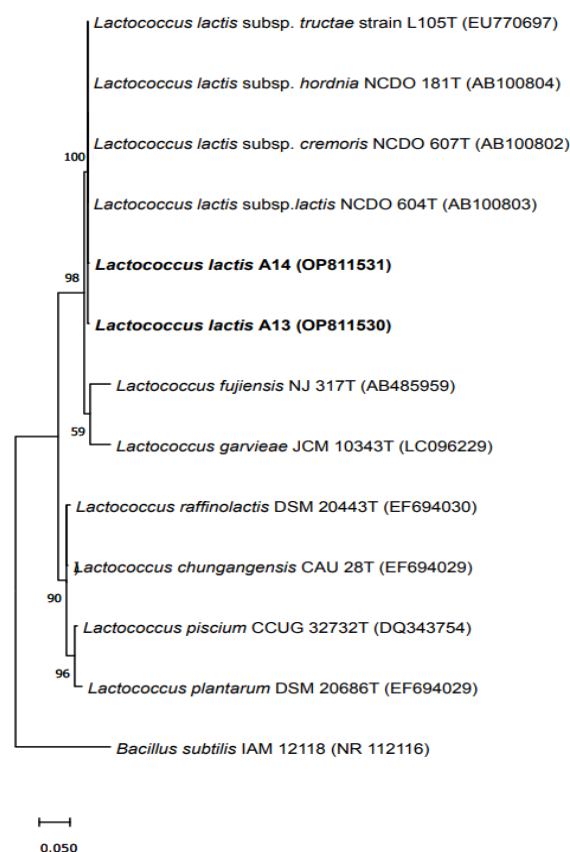


Figure 2. Maximum likelihood tree based on 16S rRNA sequences showing the phylogenetic analysis of the studied *L. lactis* isolates (bolded). Bootstrap values >50% are indicated. The scale bar displays the number of nucleotide substitutions per site. The sequence of *Bacillus subtilis* was used as an outgroup.

3.3. Metabolism of Carbon Sources

All the *L. lactis* isolates were tested for their ability to assimilate 49 carbohydrates and their derivatives using the API 50CH test (Table 2). After incubation, only 20 chemical compounds were used as media by the selected strains. All isolates of the bacteria were able to ferment glucose, lactose, ribose, fructose, mannose, galactose, N-acetyl-glucosamine, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, turanose, and potassium gluconate. Some differences were found, i.e., the strains from group I were the only bacteria that additionally assimilated glycogen, xylitol and gentiobiose, strains from group I and II additionally assimilated starch, and bacteria from group I and III utilized D-melibiose. No isolates were able to ferment glycerol, erythritol, D- and L-arabinose, D- and L-xylose, adonitol, methyl- β -D-xylopiranoside, sorbose, rhamnose, inositol, dulcitol, mannitol, sorbitol, methyl- α -D-mannopyranoside, methyl- α -D-glucopyranoside, saccharose, trehalose, inulin, D-melesitose, raffinose, lyxose, tagatose, D- and L- fucose, D- and L- arabitol, potassium 2-ketogluconate, and potassium 5-ketogluconate. The API CH test, which is a popular method for the identification and biochemical characterisation of bacteria, has often been used by researchers [5,11,13,32]. For example, Karakas-Sen [5] identified *L. lactis* and observed that all strains assimilated ribose but not inulin, which confirms our observations. In contrast, the results reported by Alharbi et al. [32] were partially different from our findings, as the authors observed that the isolates hydrolysed D-mannitol, D-sucrose, D-trehalose, D-melezitose, and D-turanose, but various patterns of catabolic activity were noted for gentiobiose, D-galactose, glucose, ribose, amygdalin, L-arabinose, and D-melibiose. In a study conducted by Sałański et al. [11], raw milk and bryndza cheese isolates assimilated 14 carbon sources and, unlike A13 and A14, they assimilated sucrose, but did not assimilate turanose and potassium gluconate, and partially only used starch and gentiobiose. Similar

to ours and other researcher studies, there was a negative bacterial response to raffinose and inulin [5,11,13,32].

Table 2. Results of carbohydrate fermentation by the *L. lactis* isolates assessed with the API 50CH test.

Carbon Source	Isolate		
	A1, A2, A4, A6, A10, A11, A13, A14, A15, A16, A18, A19, A21, A22, A24, A25	A3, A7, A8, A20, A23	A5, A9, A12, A17, A26
Glycerol	-	-	-
Erythritol	-	-	-
D-arabinose	-	-	-
L-arabinose	-	-	-
D-ribose	+	+	+
D-xylose	-	-	-
L-xylose	-	-	-
D-adonitol	-	-	-
Methyl β -D-xylopyranoside	-	-	-
D-galactose	+	+	+
D-glucose	+	+	+
D-fructose	+	+	+
D-mannose	+	+	+
L-sorbose	-	-	-
L-rhamnose	-	-	-
Dulcitol	-	-	-
Inositol	-	-	-
D-mannitol	-	-	-
D-sorbitol	-	-	-
Methyl α -D-mannopyranoside	-	-	-
Methyl α -D-glucopyranoside	-	-	-
N-acetyl-glucosamine	+	+	+
Amygdaline	+	+	+
Arbutyne	+	+	+
Esculin	+	+	+
Salicin	+	+	+
D-cellobiose	+	+	+
D-maltose	+	+	+
D-lactose	+	+	+
D-melibiose	+	-	+
D-saccharose	-	-	-
D-trehalose	-	-	-
Inulin	-	-	-
D-melezitose	-	-	-
D-raffinose	-	-	-
Starch	+	+	-
Glycogen	+	-	-
Xylitol	+	-	-
Gentiobiose	+	-	-
D-turanose	+	+	+
D-liksose	-	-	-
D-tagatose	-	-	-
D-fucose	-	-	-
L-fucose	-	-	-
D-arabitol	-	-	-
L-arabitol	-	-	-
Potassium gluconate	+	+	+
Potassium 2-ketogluconate	-	-	-
Potassium 5-ketogluconate	-	-	-

+ positive reaction, - negative reaction.

3.4. Enzymatic Activity

The API ZYM tests used for the analysis of the *L. lactis* A1-A26 strains showed three profiles of enzymatic activity, with three enzymes exhibiting positive activities: leucine arylamidase was observed in two profiles and acid phosphatase and naphthol-AS-BI-phosphohydrolase were observed in all profiles. However, most enzymes showed no or very weak activity (Table 3). The enzymatic activity of microbes is an important factor for the safety of probiotics; therefore, it is important to determine enzymatic activities of, e.g., lipase, esterase, and β -glucuronidase. The studied strains were found to have no activity of these enzymes, or this activity was weak. Similar observations were reported by Karakas-Sen et al. [5]. Abarquero et al. [8] reported the ability of *Lactococcus* isolates to produce esterases with low-medium activities. Isolates obtained by Alharbi et al. [32] showed other activities, e.g., strong valine, cystine arylamidase, N-acetyl- β -glucosaminidase, and α -mannosidase activities. In a study conducted by Nomura et al. [15], lactococci exhibited a different profile, as they produced leucine arylamidase, phosphatase, naphthol-AS-BI-phosphohydrolase, and α - and β -glucosidase, while *L. lactis* IDCC 2301 obtained by Taeok et al. [13] differed from above strains in that they produced α -chymotrypsin but not α -glucosidase.

Table 3. Enzyme activities of the *L. lactis* strains isolated from cow milk determined by the API-ZYM system.

Enzyme	Isolate		
	A1, A3, A4, A7, A8, A10, A11, A13, A14, A16, A17, A18, A20, A22, A23, A25, A26	A6, A19, A24	A2, A5, A9, A12, A15, A21
Alkaline phosphatase	1	0	2
Esterase (C4)	2	0	1
Esterase lipase (C8)	0	1	0
Lipase (C14)	1	0	1
Leucine arylamidase	3	4	2
Valine arylamidase	1	1	0
Cystine arylamidase	0	1	0
Trypsin	0	0	0
α -Chymotrypsin	1	0	1
Acid phosphatase	5	4	5
Naphthol-AS-BI-phosphohydrolase	2	2	2
α -Galactosidase	0	0	0
β -Galactosidase	0	0	0
β -Glucuronidase	0	0	0
α -Glucosidase	0	0	0
β -Glucosidase	0	0	0
N-acetyl- β -glucosaminidase	0	0	0
α -Mannosidase	0	0	0
α -Fucosidase	0	0	0

0–1—no enzymatic activity, 2–3—weak activity, 4–5—strong activity.

It is worth mentioning that the enzyme leucine arylamidase catalyses the removal of an N-terminal leucine from arylamides or p-nitroanilides [33], which is an important technological trait of strains intended to be used as starters in cheese manufacture to ensure the development of desirable flavours [34]. On the other hand, the lack of β -glucuronidase converting precarcinogens into proximal carcinogens and stimulating colonic cancer or included in the formation of toxic steroids (oestrogen) is an advantage of these strains as potential dairy cultures [13,14]. Therefore, the two A13 and A14 strains may be candidates as probiotic starters in the dairy industry.

3.5. Probiotic Potential of *Lactococcus* Strains A13 and A14

3.5.1. Acid and Bile Tolerance

An analysis of the survival properties of the probiotic strains in the presence of bile salts and an acidified environment is necessary when choosing the best probiotic strains. Probiotic cultures should not only survive but also grow in gastrointestinal conditions similar to those prevailing in the human gastrointestinal tract [35,36]. Many experiments have been carried out with the use of potentially probiotic strains that showed growth at pH 2.0–3.0 and bile salt concentrations of 0.3% and 1% [6,10,37,38].

The low pH and bile viability of the *L. lactis* A13 and A14 strains was tested to demonstrate their probiotic properties. A reduction in the number of cells at pH 2 and a greater number of cells at pH3 was observed in both the A13 and A14 strains. However, the reference strain *L. rhamnosus* GG achieved higher results at pH 2 and pH 3 (Table 4). During the cultivation of A13 at pH 3 for 3 h, there was a 2.15-log reduction in the cell count, while A14 showed a 2.84-log reduction. Similar results (a 2–3-log decrease in the cell number) were obtained by Faye et al. [39] in the same conditions. In addition, the A13 and A14 strains showed 4.73–4.91-log reductions in cell count during growth at pH 2 after a 3 h exposure. Similar results were obtained by other researchers [10], who reported a decrease in the number of cells of isolated lactococci by 4–5-log cfu/mL in an acidic environment. Moreover, both A13 and A14 and the reference *L. rhamnosus* GG were resistant to bile salts at concentrations of 0.3% and 1%. The different results of the *L. lactis* and *L. rhamnosus* GG tolerance to bile salts and acids showed that the characteristics of the tested A13 and A14 strains were unique. The obtained research results are consistent with other reports on significant differences in tolerance to bile among the LAB strains [6,12,38]. For example, in the study conducted by Galli et al. [10], only one strain of *L. lactis* MK L1 out of seven isolates tested was resistant to bile salts, while Kondrotiene et al. [37] reported that 80% of the preselected *L. lactis* strains from 33 raw and fermented milk isolates showed resistance to 1% bile salts. In turn, Sałański et al. [11] showed an approximately 50% reduction in the growth of strains IBB109 and IBB417 after 6 h of incubation in a medium containing 0.3% bile salts.

Table 4. Viability of the *Lactococcus lactis* after exposure to low pH and bile salt.

Stress Agent	Exposition Time (h)	Viability (Log CFU ¹ /mL)		
		<i>L. lactis</i> A13	<i>L. lactis</i> A14	<i>L. rhamnosus</i> GG
pH7	0	8.82 ± 0.14 ^{2a}	8.60 ± 0.13 ^a	8.54 ± 0.18 ^b
pH7	2	9.20 ± 0.21 ^b	8.95 ± 0.10 ^a	9.01 ± 0.12 ^a
pH7	3	9.33 ± 0.25 ^c	9.24 ± 0.22 ^a	9.16 ± 0.13 ^a
pH3	0	8.81 ± 0.15 ^c	8.60 ± 0.12 ^c	8.49 ± 0.15 ^a
pH3	2	7.76 ± 0.13 ^b	6.75 ± 0.12 ^b	8.67 ± 0.12 ^a
pH3	3	7.18 ± 0.15 ^a	6.40 ± 0.11 ^a	8.54 ± 0.14 ^a
pH2	0	8.85 ± 0.14 ^c	8.60 ± 0.13 ^c	8.52 ± 0.12 ^c
pH2	2	5.52 ± 0.15 ^b	4.91 ± 0.04 ^b	6.81 ± 0.15 ^b
pH2	3	4.42 ± 0.08 ^a	4.52 ± 0.03 ^a	5.31 ± 0.08 ^a
BS ³ 0.3%	0	8.80 ± 0.15 ^a	8.64 ± 0.11 ^a	8.71 ± 0.13 ^a
BS 0.3%	2	8.91 ± 0.11 ^a	8.55 ± 0.14 ^a	8.82 ± 0.11 ^{ab}
BS 0.3%	3	9.06 ± 0.13 ^a	8.45 ± 0.10 ^a	8.97 ± 0.12 ^b
BS 1%	0	8.74 ± 0.12 ^b	8.64 ± 0.12 ^a	8.79 ± 0.12 ^a
BS 1%	2	8.83 ± 0.14 ^a	8.03 ± 0.14 ^a	8.64 ± 0.11 ^a
BS 1%	3	8.97 ± 0.16 ^{ab}	7.96 ± 0.16 ^{ab}	8.78 ± 0.16 ^a

¹ CFU: colony-forming units; ² mean ± standard deviation ³ BS: bile salts. Various lowercase letters represent significantly different mean values per one strain according to a one-way ANOVA with post hoc Fisher's test ($p > 0.05$).

3.5.2. Antibacterial Activity

An important feature of a probiotic strain is the ability to inhibit the growth of pathogenic bacteria in the gastrointestinal tract. LAB have different mechanisms of antagonism and their effect on Gram-positive and Gram-negative bacteria is species- or even strain-dependent. Many researchers have identified this feature in vitro using the

well-diffusion method and various food-borne pathogenic bacteria, e.g., *Salmonella* spp., *L. monocytogenes*, *S. aureus*, *E. coli*, *B. cereus*, and others [5,28]. The two isolated strains, A13 and A14, inhibited the growth of two strains of *Salmonella* spp. most effectively but did not inhibit Gram-positive bacteria, such as *B. cereus* and *L. monocytogenes* (Table 5). The differences in the inhibition zones of *S. anatum* and *E. faecalis* suggest differences between both the studied strains. Karakas-Sen et al. [5] observed similar differences between the *L. lactis* strains. They reported that 36.365% of the strains inhibited the growth of *L. monocytogenes*, 15.15% inhibited *C. perfringens*, and 9.1% were active against *S. aureus*. In a study on *L. lactis* isolates, Alhabri et al. [32] observed different but high levels of inhibition of pathogens, such as *E. coli*, *B. subtilis*, *L. monocytogenes*, *S. aureus*, and *S. typhi*. Similarly, Rasovic et al. [29] showed differences between *L. lactis* strains in their activity against *S. aureus*; however, the strains did not inhibit Gram-negative bacteria, such as: *Escherichia* spp., *Enterobacter* spp., *Serratia* spp., and *Pseudomonas* spp. Lactococci isolated by Tsigkrmani et al. [12] from sheep milk and cheeses were not active against *L. monocytogenes*, *S. enterica* and *E. coli* O157:H7. These observations confirm the different mechanisms of antimicrobial action of *L. lactis* strains against pathogens.

Table 5. Antibacterial activity of *L. lactis* strains against different pathogens.

Pathogens	Antibacterial Activity (Inhibition Zone [mm])	
	<i>L. lactis</i> A13	<i>L. lactis</i> A14
<i>Salmonella anatum</i>	12 ± 1.1 ^{1d}	8 ± 1.3 ^d
<i>Salmonella enterica</i>	13 ± 1.0 ^e	12 ± 2.0 ^e
<i>Escherichia coli</i>	4 ± 2.3 ^a	4 ± 1.5 ^a
<i>Bacillus cereus</i>	-	-
<i>Listeria monocytogenes</i>	-	-
<i>Staphylococcus aureus</i>	7 ± 2.0 ^b	5 ± 2.5 ^b
<i>Enterococcus faecalis</i>	11 ± 1.2 ^c	7 ± 1.3 ^c

- negative (no antimicrobial activity); ¹ mean ± standard deviation. Various lowercase letters represent significantly different mean values per one strain according to a one-way ANOVA with post hoc Fisher's test ($p > 0.05$).

3.5.3. Autoaggregation/Coaggregation

The ability of bacteria to aggregate and co-aggregate determines its ability to create aggregates with possible pathogens present in the intestine. A good probiotic is believed to have these abilities higher than pathogens [40]. Strains A13 and A14, however, showed quite poor aggregation and co-aggregation abilities and needed 24 h to reach aggregation levels of 28 and 38%, respectively, and co-aggregation in the range of 28.9–45.14% depending on the combination (Table 6). *L. lactis* KC24 isolated from kimchi showed 36.15% aggregation and 29.28–74.11% co-aggregation capacities with pathogens *L. monocytogenes* and *S. aureus* after 5 h of incubation [40]. Nikolic et al. [41] in their study on this phenomenon proved that the ability to autoaggregate and co-aggregate depends on cell surface proteins and some ions.

Table 6. Autoaggregation and co-aggregation abilities of the *L. lactis* A13 and A14 isolates.

Bacteria	Autoaggregation (%)		Co-Aggregation (%)	
	5 h	24 h	5 h	24 h
<i>L. lactis</i> A13	8.52 ± 1.43 ¹	28.15 ± 0.36	-	-
<i>L. lactis</i> A14	7.49 ± 2.79	38.46 ± 3.33	-	-
<i>S. aureus</i>	12.03 ± 3.84	45.74 ± 5.73	-	-
<i>S. anatum</i>	31.81 ± 1.89	66.91 ± 0.77	-	-
A13 + <i>S. aureus</i>	-	-	13.70 ± 0.01	28.91 ± 0.06
A13 + <i>S. anatum</i>	-	-	27.80 ± 0.01	38.57 ± 0.02
A14 + <i>S. aureus</i>	-	-	15.76 ± 0.02	44.57 ± 0.01
A14 + <i>S. anatum</i>	-	-	29.39 ± 0.02	45.14 ± 0.03

¹ mean ± standard deviation; - not applicable.

3.6. Haemolytic Activity

The ability of bacteria to produce haemolysins, which are capable of lysing red blood cells, is clear evidence of their pathogenicity in humans. Therefore, analysis of this group of enzymes is crucial for food-grade bacteria [13]. Both *L. lactis* strains A13 and A14 did not show haemolytic activity, which initially may prove their non-pathogenicity, and thus their safe use in the dairy industry. Similar results were obtained by Tsigkrimini et al. [12] and Teak et al. [13].

3.7. Technological Features of *L. lactis*

3.7.1. Ability of Strains to Acidify Milk and Form a Clot

One of the factors determining the selection of A13 and A14 isolates for further research was their ability to quickly acidify milk and form a clot. During acidification at 30 °C, the pH of the milk steadily decreased over 9 h of measurements to the values of 4.51 and 5.03 for A13 and A14, respectively. pH stabilization was observed for 12 h, reaching values around 4.3 for both strains within 24 h. *L. lactis* strains isolated from sheep milk and Greek cheeses [12], similarly to isolates A13 and A14, quickly acidified milk, after 6 h they lowered the pH to 5.9 and within 6 to 12 h formed a milk clot. Similarly, *L. lactis* isolates from raw cow milk obtained by Karakas-Sen et al. [5] after 24 h of incubation acidified milk to a pH value within the range of 4.33–5.2 with an average of 4.6. In general, it can be stated that the A13 and A14 isolates are characterized by very good acidifying activity, which is a valuable technological feature.

3.7.2. Rheological Properties of Milk Clot

To assess the suitability of the A13 and A14 strains for cheese making, the clotting rate during lactic fermentation was investigated in comparison to two commercial starter cultures, S1 and S2, used in the dairy industry. Dynamic rheometry can monitor the gelation process without destruction of the gel network by measuring properties at low-strain rates [42]. Changes in complex modulus (G^*) are shown in Figure 3. Complex modulus shows the viscoelastic behaviour of the sample. The higher the G^* values, the stronger the gel [43]. The rheological analysis showed that fermentation with only one strain added to the milk yielded lower complex modulus values, compared to milk fermented with two strains. This is probably an effect of synergistic reactions between the applied strains. It is a very common phenomenon in dairy practice. Commercial production of sour cream, yoghurt, kefir, and quark relies on the addition of at least two different strains [7]. At the end of the fermentation process, the wildtype *L. lactis* A13 and A14 strains were characterized by very similar values of G^* to those exhibited by the commercial starters S1 and S2. When the strains were combined, a spectacular increase in the complex modulus value was noted. The results suggest that the wildtype strains can be successfully applied commercially for curd formation.

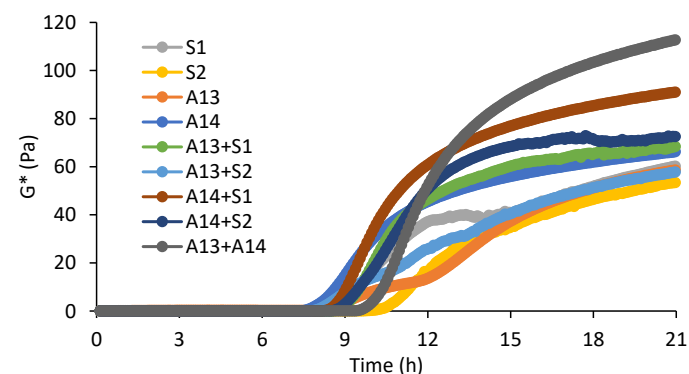


Figure 3. Changes in complex modulus during the fermentation of skimmed milk by two wildtype *L. lactis* strains, A13 and A14, in comparison to commercial starter cultures, S1 and S2, and in combined application.

3.7.3. Sensory Evaluation of Cheese

To highlight the suitability of the new *L. lactis* strains for the production of cheese with correct and/or attractive sensory characteristics, they were compared with cheeses produced by commercial starter cultures, S1 and S2, and by combined strains. The cheeses obtained by various combinations of strains (A13, A14, S1, S2, A13 + S1, A13 + S2, A14 + S1, A14 + S2, A13 + A14) are presented in Figure 4. The maximum sensory scores were awarded to cottage cheeses produced by the mixed *L. lactis* A13 + A14 strains (Figure 5A,B), i.e., 4.44 for the colour, 4.27 for the smell, 4.5 for the texture, 4.125 for the taste, and 4.5 for the moisture, followed by the strain mixtures A13 + S1 and A13 + S2. The weakest sensory results were achieved by cheeses manufactured with use of the S1 culture (3.87 for the smell, 4.25 for the texture, 3.75 for the taste, and 4.13 for the moisture; however, the colour was highly appreciated with a score of 4.65), with the S2 culture (4.71 for the colour, 3.86 for the smell, 3.87 for the texture, 3.37 for the taste, and 4.37 for the moisture), and with the A14 strain (4.74 for the colour, 3.62 for the smell, 4.25 for the texture, 3.87 for the taste, and 4.25 for the moisture). As emphasised by the panellists, the best cheese was made with the use of the newly isolated strains *L. lactis* A13 and A14; therefore, they can be used for cheese production in the future. In a study conducted by Nomura et al. [15], samples of coagulated milk inoculated with *L. lactis* isolates were organoleptically evaluated in terms of flavour by assessment of the degree of bitterness, maltiness, and sourness and the results showed a clean, acid flavour in samples inoculated with only 20% of the strains.



Figure 4. Photograph of cottage cheeses obtained with the use of *L. lactis* A13 and A14, commercial starter cultures S1 and S2 and their blends. Symbols as in Figure 4.

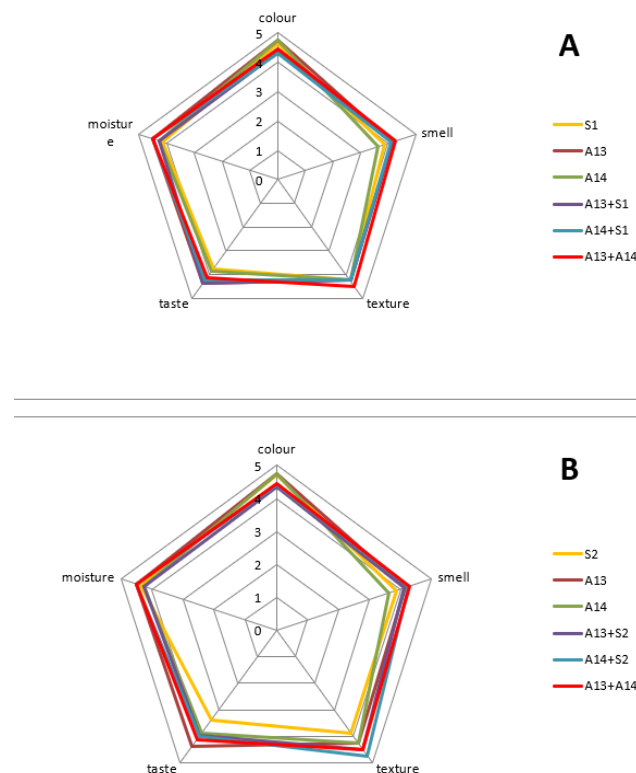


Figure 5. Sensory scores of cottage cheeses according to a 5-point scale; **(A)** cheeses with *L. lactis* A13 and A14, their blend, commercial starter culture S1, and the blend of A13 and A14 with S1, **(B)** cheeses with *L. lactis* A13 and A14, their blend, commercial starter culture S2, and the blend of A13 and A14 with S2.

4. Conclusions

L. lactis-native bacteria of milk origin may be used for cheese production thanks to their favourable technological properties. Here, 26 isolates of lactococci were screened, genetically identified, and biochemically characterized. Two of them, A13 and A14, were the most promising and were selected for further study in terms of usefulness to the dairy industry. They showed very good growth at temperatures between 30–37 °C, in medium with 6.5% NaCl and suitable enzymatic potential. They dynamically acidified milk and created milk clots separately and in mixture showing synergism similar or even better than the activity of commercial starter cultures. Cottage cheeses produced by them also obtained positive scores in sensory evaluation. Moreover, the strains showed basic probiotic features: the ability to grow in media with low pH and in the presence of bile salts, which corresponds to their ability to survive in the digestive tract. They were antagonistic against some food-borne pathogenic bacteria, such as *Salmonella* spp. and *S. aureus*, but showed poor aggregation and co-aggregation capacity with the cells of these pathogens. They met the basic safety conditions, showing no haemolytic activity. Further research into the probiotic potential and safety of the strains and their exact contribution to the formulation of the cottage cheese should be investigated.

Author Contributions: Conceptualisation, M.P. and M.K.-W.; methodology, M.P., J.W., M.K.-W., P.G. and E.S.; validation, M.P., J.W. and P.G.; formal analysis, M.P., J.W. and P.G.; investigation, M.P., J.W., M.K.-W., P.G. and E.S.; resources, M.P., and P.G.; data curation, M.P.; writing—original draft preparation, M.P., M.K.-W., and P.G.; writing—review and editing, M.P., M.K.-W. and E.S.; visualisation, M.P., and P.G.; supervision, M.P. and M.K.-W.; project administration, M.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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