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Research Article

Determination of potential probiotic properties of lactic acid bacteria isolated from colostrum milk

Pervin Soyer[1], Melek Tekgöz[2], Yağmur Tunalı[1]

[1]Anadolu University, Faculty of Pharmacy, Department of Pharmaceutical Microbiology, Eskişehir, Türkiye.
[2]Eskişehir Technical University, Faculty of Science, Department of Biology, Eskişehir, Türkiye.

✉ Pervin Soyer
pervinsoyer@anadolu.edu.tr

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ABSTRACT

Colostrum milk has been used as a source for isolating many probiotic bacteria since it contains many helpful probiotic microorganisms. Because of the huge microorganism diversity and functionality of colostrum, there is opportunity to isolate bacteria and analyze their probiotic potential. Comparing the diversity of probiotic bacteria in milk from postpartum periods is also crucial. In the current study, Lactic Acid Bacteria (LAB) cultures were isolated from cow colostrum milk. The 28 cultures were isolated, but only 2 were characterized as LAB by their colony and molecular characterization, Gram nature, catalase, antibiotics, and pepsin tolerance. As a result of molecular identification tests, the isolates were identified as *Lactobacillus casei* and *Lactobacillus paracasei* with 99-100% homology. These two isolates could survive in the presence of gastric and intestinal conditions. These isolates also showed antimicrobial and antioxidant activities. The results demonstrated that LAB species isolated from colostrum milk exhibited promising probiotic properties and seemed favorable for use in pharmaceuticals and foods.

Keywords: Colostrum, gastrointestinal tolerance, *Lactobacillus*, probiotic, postpartum milk

1. INTRODUCTION

Colostrum is the first milk produced by a female mammals (including human) after parturition. The World Health Organization suggests feeding all newborns with colostrum, a creamy yellow liquid that the mother secretes during birth and is high in many lacto-proteins [1]. It is also called postpartum or pre-milk because it is secreted by the mammary gland in the first 2-4 days of postpartum period. This fluid is essential for the newborn mammals transfer of passive immunity. Nutritionally, colostrum is rich in macronutrients (lipids, proteins, and growth factors), micronutrients (vitamins, minerals, oligosaccharides) and many unique nutritive compounds. It also plays a fundamental protection role with its valuable bioactive compound content, which includes immunoglobulins (Igs), lactoferrin (LF), lactoperoxidase, lysozyme, and cytokines. Bovine colostrum is characterised by immunoglobulin G (IgG), which is particularly important for granting passive immunity. In addition, the two most prevalent antibacterial components of colostrum are lactoferrin and lactoperoxidase. Colostrum has been utilized for the treatment and prevention of numerous infectious disorders brought on by bacteria, viruses, and protozoal pathogens due to the presence of these antimicrobial compounds. Compared to raw milk, colostrum has much more of these beneficial components.

Colostrum was utilized to cure a variety of illnesses thousands of years ago. Due to its antibacterial characteristics, doctors employed colostrum before

the discovery of antibiotics, and virologist Dr. Albert Sabin created the first polio vaccine using antibodies from cow colostrum. Because of its potent advantages as a supplementary or alternative to the medical treatment or prevention of illnesses for people of all ages, colostrum was introduced as a natural food additive. Consequently, colostrum can be applied to various products in tablet, capsule, and liquid forms to enhance the immune system, digestion, and protection against infections. Moreover, it was used commercially in food supplements in frozen, instant, or microencapsulated form. According to its useful advantages in human nutrition, colostrum is becoming more and more popular. According to latest researches, it is used to cure viral illnesses like polio, AIDS, and others and lessen the severity of their symptoms. Colostrum and its components were described by Galdino et al. [2] as a non-drug alternative to the treatment of COVID-19.

Raw colostrum contains valuable microorganisms, which are known as probiotics. Compared with milk, colostrum is a more lactose-based material that contains many bioactive compounds and also serves as a source of probiotic bacteria. Probiotics are live microorganisms that confer health benefits to the host when administered in an adequate amount [3]. Throughout the past twenty years, there have been more research showing the benefits of probiotics. As food supplements, probiotics have been shown to increase the biocompatibility and bioactivities of nutrients. Colostrum-based probiotics stimulate the immune system, control pathogen microorganisms' growth, and reduce the risk of cancer. Several studies have reported the anticarcinogenic activity of colostrum milk and its antimicrobial activity in vitro against a wide variety of bacteria and fungi. They are also known to alleviate inflammations, intestinal problems, and diarrhea [4] as well as help prevent hypercholesterolemia by deconjugating bile salts (BSH) to liberate free amino acids such as glycine [5-6]. Raw colostrum milk mainly consists of lactic acid bacteria (LAB) and *Bifidobacterium spp*. Lactic acid bacteria isolated from colostrum were 55.3% *Lactobacillus* genus [7]. There is rising interest, especially in these strains that are commonly used in food supplements due to their acclaimed health benefits. They protect against infectious diseases, improve the immune response, and reduce symptoms of irritable bowel syndrome, ulcerative colitis, allergic diseases, and atopic dermatitis associated with immunoglobulin E. The combination of probiotics' and colostrum's health advantages may help to fill new product gaps in the functional food and dietary supplement markets [8]. Only a small number of studies on probiotics made from colostrum and their positive effects have been reported, despite their wide range of therapeutic effects. The potential of probiotics of colostrum origin to exert actions in biotherapy has not been studied in detail. Due to the nutritive value of colostrum milk, this research tries to report and identify different probiotic species in the colostrum of cows and their biological activities. This research hypothesized that some colostrum-based probiotic bacteria might exhibit antimicrobial and antioxidant activities, subsequently implicating them in biotherapy as food additives. This study does not require ethics committee approval, because it does not involve an experimental process with direct animal contact. All literature sources used during the writing of this article and other similar studies confirm this situation.

2. MATERIALS AND METHODS

2.1. Sample collection

The cow colostrum milk sample was collected under hygienic conditions from dairy farm in Üçsaray-Seyitgazi (Eskişehir, Turkey) province. Sample was collected at early morning milking time on the first day of postpartum [9]. The milk sample was transferred in 250 mL pre-sterile bottles, kept in ice-box carrier and immediately brought to the Pharmaceutical Microbiology Research Laboratory to be stored at -20°C. The dairy cow species is Brown Swiss (Montofon) and known to be 4 years old.

2.2. Isolation of Lactic Acid Bacteria

LAB were isolated by serial dilution method from fresh collected cow colostrum milk. The 1 mL colostrum sample was diluted up to 10^{-6} in 9 mL of 0.9 % NaCl (Physiological Salt Water). 100 µL of

each diluted sample was inoculated on MRS (DeMan, Rogosa, Sharpe) and M17 Agar plates and incubated at 37°C for 24-48 h [10]. Following the incubation period, colonies with various morphologies were chosen, and the purity of each was evaluated using the Gram staining technique. The pure colonies were kept at -80°C in 20% glycerol. The 28 pure bacterial colonies with various morphologies conducted to the Gram-staining and catalase tests. Only 2 Gram-positive and catalase-negative colonies were subcultured for further tests.

2.3. Identification of LAB

LAB identifcation was performed by applying the 16S rRNA sequence analysis. The choromosal DNA was extracted, and the 16S rRNA (~1.5 kb) gene was amplifed by PCR procedure. 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') universal primers were used for amplification. PCR samples were examined on 1.5 % agarose gel electrophoresis and purified using the ExoSAP-IT PCR Product Cleaning Reagent (ThermoFisher Scientific) purification kit according to the manufacturer's instructions. The BigDye Terminator v3.1 Cycle Sequencing Kit and ABI 3730XL Sanger Sequencing Device (Applied Biosystems) were used for Sanger Sequencing (Applied Biosystems). The BLAST program on the NCBI website was used to examine the homology of isolate sequences [11].

2.4. Determination of simulated gastrointestinal tolerance

The tolerance to gastrointestinal conditions was determined by using pepsin at different pH levels and incubation periods. The 3 mg/mL pepsin was added to the prepared pH 2.0 and pH 3.0 PBS buffers and filtered through a 0.22 µm cellulose acetate membrane filter.

The overnight LAB isolates were centrifuged at 10000 g for 5 min at 4ºC, and bacterial pellets were washed twice with saline solution and inoculated (10^9 cfu/mL) into prepared solutions. These suspensions were incubated at 37°C for 0, 1, and 2 hours, and after the incubation periods, LAB samples were inoculated onto MRS Agar to determine cell counts by using the plate-count method [12].

2.5. Determination of antibiotic susceptibility

The disc diffusion method was used to assess the antibiotic sensitivity of LAB isolates to standard commerially cantibiotics: Ampicillin (AM 10 µg/disc, Bioanalyse), Penicillin (P 10 U/disk, Bioanalyse), Amoxicillin (AX 10 µg/disc Bioanalyse), Teicoplanine (T 30 µg/disc Bioanalyse), Streptomycin (S 10 µg/disc Bioanalyse), Chloramphenicol (C 30 µg/disc Bioanalyse), (Erythromycin (E 15 µg/disc, Bioanalyse). The overnight LAB isolates (100 µL) at 10^8 cfu/mL were inoculated onto MRS Agar, and antibiotic discs were placed. After an incubation period, at 37ºC for 18 h, the results were determined by measuring the diameter of the inhibition zones [13]. The results were evaluated in terms of susceptibile, or resistance by the CLSI (Clinical & Laboratory Standards Institute) M02-A11 (2012) scale [14].

2.6. Determination of antimicrobial activity

The overnight LAB isolates were centrifuged at 10000 g for 15 min, and the supernatant was filtered with a 0.45 µm cellulose acetate membrane filter. The antimicrobial activity of cell-free LAB isolates was determined by the disc diffusion method against 5 standard pathogens: *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 14028, *Escherichia coli* ATCC 35218, and *Campylobacter jejuni* ATCC 33560 [15]. The standard test bacteria cultures (10^8 cfu/mL) were inoculated onto MHA (Mueller Hinton Agar) and after being allowed to solidify, 6 mm wells were opened. The LAB cell-free supernatants (100 µL) were added into the wells. After 37ºC-18 h incubation period, inhibition zones were measured.

2.7. Determination of antioxidant activity by DPPH assay

The radical scavenging activity of the cell-free supernatants was determined by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay [16]. The 25 µL of dilutions of the cell-free supernatants and standards

(gallic acid and ascorbic acid) were added to 975 µL of DPPH (0.025 mg/mL). The combination was then left at room temperature in a dark place for 30 minutes. Each cell-free supernatant's percentage of DPPH radical-scavenging activity was determined using the following equation after the absorbance at 517 nm was measured: DPPH scavenging effect (%)=A0-A1/A0 × 100. A0=The absorbance of control and A1=The absorbance of standard.

3. RESULTS AND DISCUSSION

3.1. Isolation and identification of LAB

A total of 28 pure bacteria were isolated from colostrum milk samples. Only 2 isolates were Gram positive, bacilli shaped, and catalase-negative. These isolates were regarded as potential LAB isolates for testing. All 2 isolates were identified by the 16S rRNA sequencing method. By using BLAST software in the NCBI site for molecular identification, these 2 isolates showed 99-100% similarity with: C1 coded isolate is *Lactobacillus casei* (Accession Number: OP000865.1), C2 coded isolate is *Lactobacillus paracasei* (Accession Number: ON631824.1). Haghshenas et al., also reported these same strains that were isolated from different regions colostrum milks [17]. While a small number of studies have shown that breastfeeding exposes a baby to a variety of commensal LAB strains and encourages their growth and colonization, not much is known about the microbial content of human colostrum [18]. In this research, *L. paracasei* and *L.casei* were the *Lactobacillus* species obtained from the milk colostrum. In the literature, the isolates that identified by 16S rRNA sequencing: *Lactobacillus casei, L. plantarum, L. pentosus, L. kefiri, L. gasseri,* and *L. paracasei* species were generally isolated from cow colostrum with 99.8% similarity to those of the reference strains [19].

3.2. Determination of simulated gastrointestinal tolerance

The isolates were subjected to pepsin as a basic test to determine their capacity to survive passing through the gastrointestinal process. To establish sufficient numbers of active and live bacteria throughout the gastrointestinal tract, LAB isolates should be acid-resistant. In the present research, LAB isolates' viable cell counts significantly decreased when they were exposed to simulated gastric juice with a low pH. The results obtained for simulated gastric juice are given in Figure 1 (a, b). Although they retained greater viable cell counts at pH 2 compared to pH 3, isolates C1 and C2 were significantly distinct. Similar findings were made by Angmo et al., who showed that in vitro incubation at pH 2 caused a noticeably lower survival rate [20]. Also in the study of Liu et at., LAB isolates were reported to transport tolerance testing in artificial gastric juice at pH 2.5 and only *L. rhamnosus* and *L. plantarum* showed similar survival rates with current research [15].

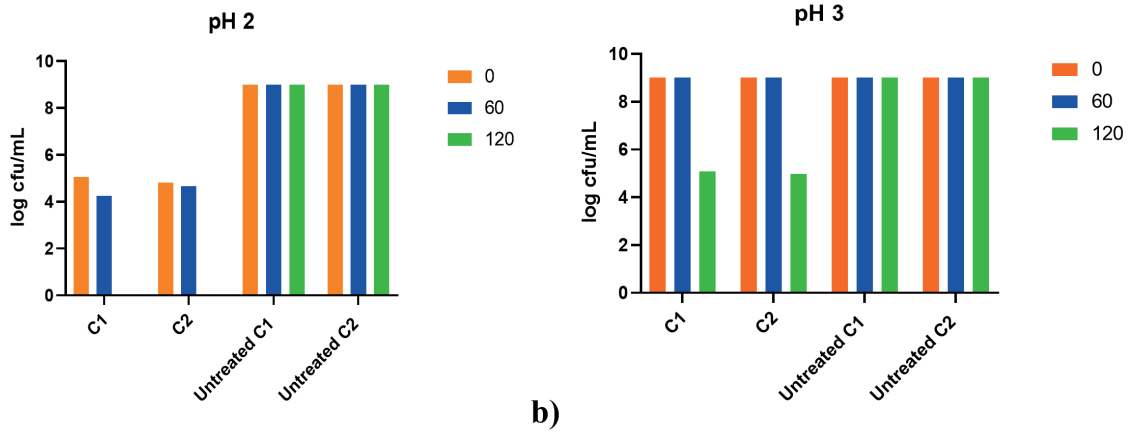

Figure 1. Gastrointestinal viability results (log cfu/mL)

The peak bacteria viability was observed at pH 3 in the simulated gastric juice. When compared with pH 2, after 120 minutes, maximum log reduction were observed for all isolates. In the literature, maximum 2 log cfu/mL reduction survival rate in gastric juice at pH 2.5-3 for 1-2 h is the standard for identifying potentially probiotic organisms [21]. The results with untreated C1 and C2 isolates reveal stabile at 9 log cfu/mL. At least, 20-40% of tests on mice show that *L. casei* and *L. paracasei* can live in the physiological conditions of the stomach and duodenum following oral administration [22].

3.3. Determination of antibiotic susceptibility

The isolated LAB strains were observed for resistance to antibiotics for safety concerns. Two functional groups of standard antibiotics are generally recommended in EFSA (European Food Safety Authority) guidelines for appropriate selection. These groups include inhibitors of cell wall synthesis (ampicillin, amoxicillin, teicoplanin, and penicillin) and inhibitors of protein synthesis (chloramphenicol, erythromycin, and streptomycin) [23]. In this research, the tested 2 isolates were susceptible to penicillin and ampicillin. These antibiotics are best known as effective inhibitors against all LAB strains. The LAB isolates were resistant to teicoplanin, chloramphenicol, erythromycin, and streptomycin. All results are given in Table 1.

Table 1. Antibiotic susceptibility results

	C1	C2
Chloramphenicol (30 µg)	R	R
Amoxicillin (10 µg)	S	R
Streptomycin (10 µg)	R	R
Ampicillin (10 µg)	S	S
Teicoplanine (30 µg)	R	R
Erythromycin (15 µg)	R	R
Penicillin (10 µg)	S	S

*R: Resistant, *S: Susceptible

3.4. Determination of antimicrobial activity

All 2 LAB isolates showed inhibition against *S.aureus*. Only *L. casei* showed antimicrobial activity against both Gram-positive (*S. aureus*) and Gram-negative (*S. typhimurim, P. aeruginosa,* and *C. jejuni*) standard pathogens (Table 2). Forestier et al., also reported that the *L. casei* have antibacterial activity against gastrointestinal pathogens [24].

And ampicillin was used as a standard antibiotic. The capacity of the LAB isolates to suppress carbohydrate fermentation is likely connected to the production of organic acids as a result of that process. Moreover, the creation of antimicrobial compounds such bacteriocins and organic acids aids LAB in its battle with the host organism for nutrients and safeguards the site of action from dangerous bacteria [25]. The antibacterial action of *Lactobacillus* strains is assumed to be caused by the release of a variety of antipathogen chemicals, including bacteriocins, biosurfactants, H_2O_2, and organic acids (hydrochloric, lactic, and acetic acids) [26].

3.5. Determination of antioxidant activity by DPPH assay

Probiotics are thought to be a new, effective source of antioxidants. In this research, antioxidant activity results were found ranged from 49.08±3.30 % (*L.*

Table 2. Antimicrobial activity of cell-free supernatants of LAB strains against standard microorganism as inhibition zone

Isolate Code – Standard Microorganisms	S. aureus	P. aeruginosa	C. jejuni	S. typhimurium	E. coli
C1	32+1.2 mm	5+0.6 mm	9+0.3 mm	8+0.9 mm	-
C2	30+1.8 mm	-	-	-	-
Ampicillin	20 mm	8 mm	10 mm	24 mm	14 mm

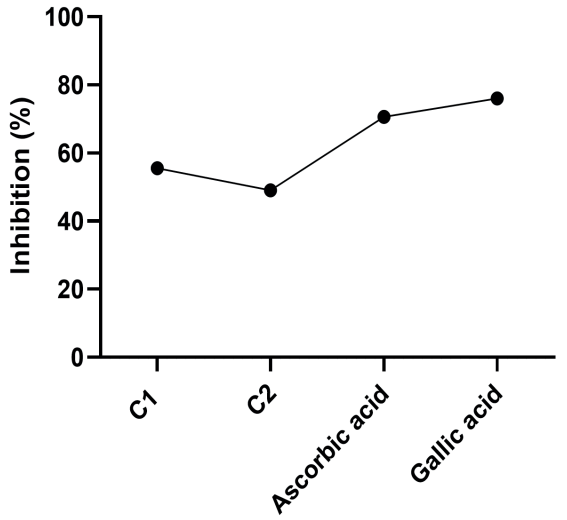

Figure 2. Antioxidant activity of cell-free supernatants of LAB isolates as percent inhibition

paracasei) to 55.51±1.7 % (*L.casei*) as shown in Figure 2. These results proved that the cell-free supernatants have good antioxidant potential when compared to standard substances (ascorbic and gallic acid). Some *Lactobacillus* species, used in the diet or as food supplements or pharmaceuticals, are known for their antioxidant effects [27]. Moreover, it has been proposed that some probiotics enhance the function of antioxidant enzymes or regulate circulatory oxidative stress, shielding cells from damage brought on by carcinogens [28]. Through the release of a peptide that can prevent oxygen radicals, recent studies have shown that *Lactobacillus* species can function as antioxidants [29].

4. CONCLUSION

Cow colostrum milk was identified as a novel source of lactic acid bacteria with the isolation of *L. casei* and *L. paracasei.* These isolates were characterized with regards to simulated gastrointestinal tolerance and antibiotic resistance. In this research, isolated LAB strains were explored by their antimicrobial and antioxidant activities as safe biotherapeutics, providing an alternative to drugs or chemicals. This research showed that *Lactobacillus casei* and *Lactobacillus paracasei* isolates can be used as single probiotic or as a contributor of synbiotic in formulations. The main difference of this research from other studies reported in scientific literature is that, LAB species were isolated for the first time from the colostrum milk of a Montofon cow species of Turkish origin, and determined by their potential probiotic properties. But, more studies are required to prove their true potential and usage areas. This phenomenon explains the growing interest in finding novel sources of probiotics that can be used in pharmaceuticals and foods.

Ethical approval

Not applicable, because this article does not contain any studies with human or animal subjects.

Author contribution

Concept: PS, MT; Design: PS; Supervision: PS, MT, YT; Materials: PS, MT; Data Collection and/or Processing: PS, MT; Analysis and/or Interpretation: PS, MT; Literature Search: PS, MT; Writing: PS, MT, YT; Critical Reviews: PS, MT, YT.

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Conflict of interest

The authors declared that there is no conflict of interest.

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