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**ORIGINAL RESEARCH ARTICLE**

# Screening of Lactic Acid Bacteria for Their Antimicrobial Activity against Pathogenic Bacteria

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**ABSTRACT**

Lactic acid bacteria (LAB) are those that produce lactic acid as the sole product or major acid from the energy yielding fermentation of sugars. They can be broadly defined as Gram positive, anaerobic, microaerophilic or aerotolerent bacteria, either rod or coccus, catalase negative and fastidious in their growth. In the present study, lactic acid bacterial isolates were screened for its antimicrobial activity against human pathogenic bacteria. Totally, 10 LAB isolates and identified as *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* sub sp. *bulgaricus*, *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactococcus lactis* sub sp. *lactis*, *Lactococcus lactis* sub sp. *cremoris*, *Streptococcus thermophilus*, *Leuconostoc* sp., *Pediococcus acidilactici* and *Enterococcus faecalis*. *Lactobacillus acidophilus* was found relatively dominating species of cow milk. The antimicrobial activity of LAB was estimated by Agar spot method, well diffusion assay and disc diffusion method. The extracts of ten-isolated LAB gave zones of inhibition against the indicator food pathogenic strains such as *Escherichia coli*, *Salmonella typhi*, *Shigella*, *Staphylococcus aureus*, *Enterobacter* and *Listeria monocytogenes.*

**Key words:** Lactic acid bacteria, pathogenic bacteria, Agar spot method, Well diffusion assay and Disc diffusion method.

## 1. INTRODUCTION

Lactic acid bacteria (LAB) occur naturally in several raw materials like milk, meat and flour used to produce foods [1]. LAB are used as natural or selected starters in food fermentations in which they perform acidification due to production of lactic and acetic acids flavour, protection of food from spoilage and pathogenic microorganisms by LAB is through producing organic acids, hydrogen peroxide, diacetyl [2], antifungal compounds such as fatty acids [3] or phenullactic acid [4] and bacteriocins [5]. LAB play an important role in food fermentation as the products obtains with their aid is characterized by hygienic safety, storage stability and attractive sensory properties. Many bacteria of different taxonomic branches and residing in various habitats produce antimicrobial substances that are active against other bacteria. Both Gram negative and Gram positive bacteria produce bacteriocins.

Lactic acid bacteria (LAB) consist of a number of bacterial general within the *Phylum fumicutes*. The general *Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Milissococcus, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* are recognized as lactic acid bacteria [6] non-sporulating, catalase negative [7] devoid of cytochromes but aero tolerant, fastidious, acid tolerant and ferment carbohydrates into energy and lactic acid depending on the organics, metabolic pathways differ when glucose is the main carbon source.

The ability of the Lactic acid bacteria to prevent and cure a variety of diseases has lead to the coining of the term probiotics or pro-life. The most important role of lactic acid bacteria is its protective role against infections and colonization of pathogenic microorganisms in the digestive track. In most of the cases inoculums passively transits the gastrointestinal track. The probiotics can influence the unspecific immunity, which consists of T- lymphocytes and B-lymphocytes. The increase in the specific immune response corresponds with the activity of B and T-lymphocytes, which leads to an increase of interleukin and the level of circulating antibodies.

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Members of these genera *Lactobacillus* plays an essential role in the fermentation of food and feed. The most important characteristics of the lactic acid bacteria are their ability to ferment sugars to lactic acid. This may desirable in making products and these organisms have been isolated and screened by using fermented foods such as curd, buttermilk, cheese and yoghurt. Different antimicrobials such as lactic acid, acetic acid, hydrogen peroxide, carbon-di-oxide and bacteriocins produced by these bacteria can inhibit pathogenic and spoilage microorganisms extending the shelf-life and enhancing the safety of food products.

## 2. MATERIALS AND METHODS

### 2.1. Collection of samples

The samples of milk, dahi, sausage, yoghurt and meat, were collected randomly from local markets of Cuddalore and were brought to the laboratory of the Department of Food technology for further analysis.

### 2.2. Isolation and identification of LAB

For microbiological analysis, 1 ml of milk and 20 gm of dahi, yoghurt, sausage and meat was aseptically transferred to 90 ml of sterile physiological saline (0.9% NaCl) (warmed to 45°C for cream) and mixed well. Dahi samples were prepared by transferring twenty grams of aseptically weighed sample to 100 ml sterile 2% (w/v) sodium citrate solution at 45 to 50°C and homogenized for 3 min. Decimal dilutions of the homogenates were prepared with sterile 0.85% (w/v) sodium chloride and were plated on media most suitable for isolation of LAB. Thirty to fifty colonies per sample were randomly taken from both M17 and MRS (30°C and 45°C) agar plates corresponding to the highest dilution at which growth occurred. Cell morphology of all isolates of LAB was determined by microscopy (Olympus U-RF L-T, BX51, GmbH, Hamburg, Germany). After Microscopic observations, the colonies were sub-cultured to purity on MRS or M17 medium for rods and cocci, respectively. Gram positive and catalase negative isolates were frozen at -20°C and -80°C in M17 (for cocci) and in MRS (for rods) both containing 15% of glycerol (v/v) [8].

### 2.3. Screening of LAB for their antimicrobial properties

The LAB cultures isolated in this study were screened for their antagonistic activity against bacteriocin sensitive strains by using Yang *et al.* [9] method. An overnight culture of each isolate were grown in MRS broth at 37°C and standardized to an optical density of 0.5 at a wavelength of 600 nm (spectrophotometer). One percent of standardized culture was used to inoculate MRS broth. After incubation at 37°C for 24 hrs, cells were removed by centrifugation at 10,000 rpm for 15 min. The pH of one portion of supernatant was adjusted to 7.0 and filtered through 0.22□m membranes. The filtrates of both pH and non-pH adjusted were used to evaluate antimicrobial activity method. Positive results were recorded when the zone of inhibition of at least 1 mm around the wells was observed.

#### 2.3.1. Agar spot method

Antimicrobial activity was confirmed by using the Agar spot test method as described by Eamanu *et al.* [10]. Seven ml of sterile BH1, soft agar was coded to 47°C and mixed with 10 □l of a cell suspension of bioassay strains (over night cultures). The soft agar was then poured over the agar plates and cooled at room temperature for 30 min. After the plates were solidified make 5 ml of cultures free supernatant of test organism. The plates were incubated at 37°C for 18-24 hrs and examined for the presence of clear zone of inhibition of 2 mm or more around the spot.

#### 2.3.2. Well diffusion assay

The antimicrobial activity of the isolated LAB (cell free filtrate) against (*Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus cereus*, *Enterobacter cloacae*, *Shigella* and *Salmonella typhi*), that obtained from MTCC was performed by the well diffusion assay. The pathogenic test bacteria were incubated in Nutrient broth at appropriate temperature for 24 hrs petridishes containing 20 ml of Muller Hinton agar were prepared previously and inoculated with 0.1 ml of 24 hrs broth culture of pathogenic bacteria. Once solidified the dishes were stored for 2 hrs in a refrigerator. Four wells were made and filled using different concentration like 25 □l, 50 µl, 75 µl, 100 µl of cell-free filtrate and the petridishes were incubated at 37°C for 24 hrs. Then the diameter of the inhibition zone was measured with calipers in mm. The antimicrobial activity was determined by measuring the clear zone around the wells.

#### 2.3.3. Disc diffusion method

Five sterile paper blank discs were placed on the agar plate which was inoculated by indicator strains and different concentration of the filtered supernatant of lactic acid bacteria were applied plates were incubated and observed for zones of inhibition.





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## 3. RESULTS AND DISCUSSION

Total 10 LAB isolates were identified from the 22 isolates obtained from raw milk. The species identified were, *Lactobacillus acidophilus* (18%), *Lactobacillus delbrueckii* sub sp. *bulgaricus* (9%), *Lactobacillus plantarum* (4%), *Lactobacillus fermentum* (9%), *Lactococcus lactis* sub sp. *lactis* (14%), *Lactococcus lactis* sub sp. *cremoris* (4%), *Streptococcus thermophilus* (14%), *Leuconostoc* sp. (9%), *Pediococcus acidilactici* (14%) and *Enterococcus faecalis* (4%). *Lactobacillus acidophilus* was found relatively dominating species of cow milk.

Lactic acid bacteria (LAB) particularly those belonging to beneficial and non-pathogenic bacteria (*Lactococcus, Lactobacillus, Leuconostoc, Streptococcus* and *Oenococcus*) have traditionally been used in the food industry. They also play an essential role in the dairy industry due to the tremendous level of human consumption of several important fermented products, mainly cheese and acidified or fermented milks [11]. Total of 88 lactic acid bacterial cultures were isolated from five tested samples. These included 41% *Lactobacillus* sp., 18% *Lactococcus* sp., 11% *Streptococcus thermophilus,* 9% *Leuconostoc* sp., 15% *Pediococcus acidilactici* and 7% *Enterococcus faecalis* which suggest that dahi and raw milk harbor highest number of *Lactobacillus acidophilus* followed by *Lactococcus lactis* sub sp. *lactis. Leuconostoc* species was found in low frequency is probably due to their inability to compete with other LAB in mixed cultures environment [12]. The strains were phenotypically characterized on the basis of their morphological, cultural, physiological and biochemical characteristics by the procedure described in Bergey's manual and Collins and Lyne [13].

Yeasts appear to be commonly associated with traditional fermented dairy products and have been reported in several studies [14,15,16]. Isono *et al.* [17] reported occurrence of yeasts in seven of 10 samples of traditional fermented milk in northern Tanzania with the mean counts ranging from 6.0 to 8.0 log 10 cfu $ml^{-1}$. In the current study, yeast counts ranged from 4.3 to 7.4 10 cfu $ml^{-1}$. It was evident from the result that the lactic acid bacteria dominated the microbial flora of dahi followed by raw milk. It might be due to the reason that two specific media. MRS and M-17 agar were used to study the morphological characteristics of rods and cocci isolates, respectively. This selective media allows only specific type of microorganisms to grow therefore the ability of bacterial species to grow on specific media is regarded as an important characteristic in identification. MRS and M17 media are the best suitable media for the isolation of lactic acid bacteria as reported earlier by Ghoddusi [18]. The least microbial population was recorded in the sausage sample.

In the present investigation, *Lactobacillus delbrueckii* sub sp. *bulgaricus, Lactobacillus acidophilus, Lactococcus lactis* sub sp. *lactis* and *Streptococcus thermophilus* were observed as dominant microflora in the dahi sample. The presence of such bacteria has been reported in earlier studies [19]. Moreover, it was further observed that all the isolated bacteria from indigenous dahi were thermophillic and mesophillic in nature. This diversity of species is relative and dependent primarily on the nature of material isolated and different criteria used for each study as reported by Masud *et al.* [20]. In the milk sample out of 22 LAB, *Lactobacillus acidophilus* was dominant followed by *Lactococcus lactis* sub sp. *lactis* and *Streptococcus thermophilus.* All the *Lactococcus* isolates were identified as *Lactococcus lactis.* Several studies elsewhere reported that *Lactococcus lactis* was more frequently isolated from raw milk samples.

Raw milk is one of the primary sources to isolate LAB producing bacteriocins active against *Listeria monocytogenes.* This result confirms the high incidence of bacteriocin – producing lactic acid bacteria in milk products reported in other studies [21]. More importantly, it also seems that LAB predominate in raw milk samples universally and the variations in microflora seem primarily due to geographical, environmental and milk compositional differences among different milk species. Out of five samples meat comes to third place. In meat, totally 16 LAB were isolated. Among the 16 LAB, *Lactobacillus acidophilus* is dominant followed by *Lactobacillus delbrueckii* sub sp. *bulgaricus* and *Streptococcus thermophilus.* Ennahar *et al.* [22] reported that the most bacteriocin producing strains have been isolated from meat products. Compared to yoghurt, sausage reported least LAB count 11 out of 88. Among the five samples 18% *Lactobacillus acidophilus* isolated in this study, 16% *Lactococcus lactis* sub sp. *lactis* and 15% *Pediococcus acidilactici* followed by 12% *Lactobacillus delbrueckii* sub sp. *bulgaricus.* Due to this high prevalence, we select only three LAB





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for consortium development. Only 16 strains of *Lactobacillus acidophilus* recorded in the present study. These strains are considered to produce higher titrable acidity and result in the production of low pH that may be considered objectionable. However, the results of studies reported that these strains have the ability to produce bacteriocins.

The identified LAB strains show antagonistic activity against six food pathogens by production of bacteriocin. Maximum production of bacteriocin was obtained in MRS broth containing at least 1.2% glucose or xylose. Also, MRS medium with 1% NaCl found that the antibacterial activity increased. The inhibitory activity was maximal at the beginning of the stationary phase and remained stable long after growth had ceased, even in the presence of the producer cells. Zone inhibition of six food pathogens against supernatant of LAB by Agar spot method, well diffusion assay and disc diffusion method was measured. The extracts of ten-isolated LAB gave zones of inhibition against the indicator food pathogenic strains such as *Escherichia coli, Salmonella typhi, Shigella, Staphylococcus aureus, Enterobacter* and *Listeria monocytogenes.* Among the ten LAB, the strongest diameter zones (14-16 mm) obtained with the extracts of *Lactobacillus acidophilus, Lactococcus lactis* sub sp. *lactis* and *Pediococcus acidilactici* against *Listeria monocytogenes, Enterobacter, Staphylococcus aureus, Salmonella* and *Escherichia coli.* Smallest or week diameter zones (6-9 mm) of LAB are *Streptococcus thermophilus* and *Lactobacillus plantarum* and the intermediate diameter zones (10-13 mm) of LAB are *Str. thermophilus* and *Lactobacillus fermentum.* Resistant pathogen growth was obtained with the extracts of *Leuconostoc* sp. and *Lactococcus lactis* sub sp. *cremoris* (**Table 1 to Table 7**). The strains which showed the largest zone of growth inhibition was selected for further strain developmental studies.

Ten lactic acid bacteria were selected and allowed for antibacterial activity against seven different bacterial pathogens which are usually present in food and can cause food borne illnesses in human being. The bacterial pathogens selected were *Listeria monocytogenes, Bacillus cereus, Escherichia coli, Enterobacter, Salmonella typhi, Shigella* and *Staphylococcus aureus.* The inhibition zones of between 0.5 – 13.0 mm in diameter indicator organisms as reported by Enan *et al.* [23]. However, it can only be classified as being between non-inhibition and moderate inhibition, indicating a relatively narrow antimicrobial spectrum, this finding may be supported with [24], that all the *Lactobacillus* isolates obtained from Turkish dairy products have antimicrobials activity against *Staphylococcus aureus* and *Escherichia coli.* The result indicated the present strains seemed to the antagonistic activity against seven pathogens in the order of *Escherichia coli, Salmonella typhi, Enterobacter, Staphylococcus aureus, Listeria monocytogenes, Bacillus cereus* and *Shigella.* The study had proved the possibility of using this strain as a biopreservatives or a probiotic.

Despite the high number of bacteriocin producing LAB isolated and characterized so far, further search for new strains belonging to all genera of LAB having different spectra of action and isolated from different environments is worthwhile. According to Klaenhammer [25], 99% of all bacteria may make at least one bacteriocin. Bacteriocin are antimicrobial agents produced by bacteria which are active against closely related bacteria as claimed by Klaenhammer [26]. They have been proved active against many other bacteria also including pathogens described by Flythe and Russell [27]. Hence, they may be used as probiotic or as biopreservatives especially in the acid fermentation of food.

**Table 1: Screening of LAB for antibacterial activity against *E. coli***

| S. No | Lactic acid bacteria | Diameter of inhibitory zone | | | Inference |
|---|---|---|---|---|---|
| | | Agar spot method (mm) | Blank disc method (mm) | Agar well diffusion method (mm) | |
| 1 | *Lb. acidophilus* | ++ | ++ | ++ | S |
| 2 | *Lb. delbrueckii* ssp. *bulgaricus* | ++ | +++ | ++ | S |
| 3 | *Lb. plantarum* | + | + | + | M |
| 4 | *Lb. fermentum* | +++ | ++ | + | M |
| 5 | *Lactococcus lactis* ssp. *lactis* | +++ | +++ | +++ | S |
| 6 | *Lc. lactis* ssp. *cremoris* | – | – | – | R |
| 7 | *Str. thermophilus* | – | – | – | R |
| 8 | *Leuconostoc sp.* | – | – | – | R |
| 9 | *P. acidilactici* | ++ | ++ | ++ | S |
| 10 | *Ec. faecalis* | + | + | + | M |

*Diameter of the inhibition zone: (+) weak (6 – 9 mm), (++) intermediate (10 – 13 mm), (+++) strong (14 – 16 mm), (–) no growth*





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**Table 2: Screening of LAB for antibacterial activity against *Salmonella typhi***

| S. No | Lactic acid bacteria | Diameter of inhibitory zone | | | Inference |
|---|---|---|---|---|---|
| | | Agar spot method (mm) | Blank disc method (mm) | Agar well diffusion method (mm) | |
| 1 | *Lb. acidophilus* | ++ | ++ | ++ | S |
| 2 | *Lb. delbrueckii* ssp. *bulgaricus* | ++ | +++ | ++ | S |
| 3 | *Lb. plantarum* | + | + | + | M |
| 4 | *Lb. fermentum* | – | – | – | R |
| 5 | *Lactococcus lactis* ssp. *lactis* | +++ | +++ | +++ | S |
| 6 | *Lc. lactis* ssp. *cremoris* | + | + | ++ | S |
| 7 | *Str. thermophilus* | ++ | +++ | ++ | S |
| 8 | *Leuconostoc lactis* | ++ | ++ | +++ | S |
| 9 | *P. acidilactici* | + | + | + | M |
| 10 | *Ec. faecalis* | ++ | + | + | M |

*Diameter of the inhibition zone: (+) weak (6 – 9 mm), (++) intermediate (10 – 13 mm), (+++) strong (14 – 16 mm), (–) no growth*

**Table 3: Screening of LAB for antibacterial activity against *Shigella***

| S. No | Lactic acid bacteria | Diameter of inhibitory zone | | | Inference |
|---|---|---|---|---|---|
| | | Agar spot method (mm) | Blank disc method (mm) | Agar well diffusion method (mm) | |
| 1 | *Lb. acidophilus* | + | + | + | M |
| 2 | *Lb. delbrueckii* ssp. *bulgaricus* | ++ | ++ | + | S |
| 3 | *Lb. plantarum* | + | + | ++ | S |
| 4 | *Lb. fermentum* | + | + | + | M |
| 5 | *Lactococcus lactis* ssp. *lactis* | – | – | – | R |
| 6 | *Lc. lactis* ssp. *cremoris* | _ | _ | - | R |
| 7 | *Str. thermophilus* | – | – | – | R |
| 8 | *Leuconostoc lactis* | _ | _ | _ | R |
| 9 | *P. acidilactici* | + | + | + | M |
| 10 | *Ec. faecalis* | _ | _ | _ | R |

*Diameter of the inhibition zone: (+) weak (6 – 9 mm), (++) intermediate (10 – 13 mm), (+++) strong (14 – 16 mm), (–) no growth*

**Table 4: Screening of LAB for antibacterial activity against *Staphylococcus aureus***

| S. No | Lactic acid bacteria | Diameter of inhibitory zone | | | Inference |
|---|---|---|---|---|---|
| | | Agar spot method (mm) | Blank disc method (mm) | Agar well diffusion method (mm) | |
| 1 | *Lb. acidophilus* | ++ | ++ + | +++ | S |
| 2 | *Lb. delbrueckii* ssp. *bulgaricus* | +++ | ++ | ++ | S |
| 3 | *Lb. plantarum* | ++ | + | + | S |
| 4 | *Lb. fermentum* | ++ | ++ | ++ | S |
| 5 | *Lactococcus lactis* ssp. *lactis* | +++ | +++ | +++ | S |
| 6 | *Lc. lactis* ssp. *cremoris* | + | + | ++ | S |
| 7 | *Str. thermophilus* | ++ | ++ | ++ | S |
| 8 | *Leuconostoc lactis* | + | +++ | ++ | S |
| 9 | *P. acidilactici* | +++ | +++ | +++ | S |
| 10 | *Ec. faecalis* | +++ | +++ | +++ | S |

*Diameter of the inhibition zone: (+) weak (6 – 9 mm), (++) intermediate (10 – 13 mm), (+++) strong (14 – 16 mm), (–) no growth*

**Table 5: Screening of LAB for antibacterial activity against *Enterobacter***

| S. No | Lactic acid bacteria | Diameter of inhibitory zone | | | Inference |
|---|---|---|---|---|---|
| | | Agar spot method (mm) | Blank disc method (mm) | Agar well diffusion method (mm) | |
| 1 | *Lb. acidophilus* | +++ | +++ | +++ | S |
| 2 | *Lb. delbrueckii* ssp. *bulgaricus* | _ | _ | _ | R |
| 3 | *Lb. plantarum* | + | + | + | M |
| 4 | *Lb. fermentum* | + | + | + | M |
| 5 | *Lactococcus lactis* ssp. *lactis* | ++ | ++ | ++ | S |
| 6 | *Lc. lactis* ssp. *cremoris* | _ | _ | _ | R |
| 7 | *Str. thermophilus* | ++ | ++ | ++ | S |
| 8 | *Leuconostoc lactis* | +++ | +++ | +++ | S |
| 9 | *P. acidilactici* | +++ | +++ | +++ | S |
| 10 | *Ec. faecalis* | +++ | +++ | +++ | S |

*Diameter of the inhibition zone: (+) weak (6 – 9 mm), (++) intermediate (10 – 13 mm), (+++) strong (14 – 16 mm), (–) no growth*

**Table 6: Screening of LAB for antibacterial activity against *Listeria monocytogenes***

| S. No | Lactic acid bacteria | Diameter of inhibitory zone | | | Inference |
|---|---|---|---|---|---|
| | | Agar spot method (mm) | Blank disc method (mm) | Agar well diffusion method (mm) | |
| 1 | *Lb. acidophilus* | +++ | +++ | +++ | S |
| 2 | *Lb. delbrueckii* ssp. *bulgaricus* | + | + | ++ | S |
| 3 | *Lb. plantarum* | + | + | ++ | S |
| 4 | *Lb. fermentum* | ++ | +++ | ++ | S |
| 5 | *Lactococcus lactis* ssp. *lactis* | +++ | +++ | +++ | S |





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| | | | | | |
|---|---|---|---|---|---|
| 6 | *Lc. lactis* ssp. *cremoris* | + | + | ++ | S |
| 7 | *Str. thermophilus* | +++ | +++ | +++ | S |
| 8 | *Leuconostoc lactis* | _ | _ | _ | R |
| 9 | *P. acidilactici* | + | ++ | ++ | S |
| 10 | *Ec. faecalis* | +++ | +++ | +++ | S |

*Diameter of the inhibition zone: (+) weak (6 – 9 mm), (++) intermediate (10 – 13 mm), (+++) strong (14 – 16 mm), (–) no growth*

**Table 7: Screening of LAB for antibacterial activity against *Bacillus cereus***

| S. No | Lactic acid bacteria | Diameter of inhibitory zone | | | Inference |
|---|---|---|---|---|---|
| | | Agar spot method (mm) | Blank disc method (mm) | Agar well diffusion method (mm) | |
| 1 | *Lb. acidophilus* | + | ++ | +++ | S |
| 2 | *Lb. delbrueckii* ssp. *bulgaricus* | ++ | +++ | +++ | S |
| 3 | *Lb. plantarum* | ++ | ++ | +++ | S |
| 4 | *Lb. fermentum* | ++ | ++ | + | S |
| 5 | *Lactococcus lactis* ssp. *lactis* | + | ++ | ++ | S |
| 6 | *Lc. lactis* ssp. *cremoris* | _ | _ | _ | R |
| 7 | *Str. thermophilus* | ++ | ++ | ++ | R |
| 8 | *Leuconostoc lactis* | + | ++ | + | S |
| 9 | *P. acidilactici* | + | + | + | S |
| 10 | *Ec. faecalis* | +++ | +++ | +++ | S |

*Diameter of the inhibition zone: (+) weak (6 – 9 mm), (++) intermediate (10 – 13 mm), (+++) strong (14 – 16 mm), (–) no growth*

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