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ANTIMICROBIAL SUBSTANCE FROM A DAIRY LACTOBACILLUS STRAIN

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Lactobacillus johnsonii strain La1 isolated from commercial yogurt Lc1 produced Nestle-Mis Co. excretes a substance with antagonistic activity against a variety of pathogenic and opportunist pathogenic microoorganisms, and some lactic acid cocci, but doesn't inhibit other lactobacilli.

Antimicrobial substance from this strain is active at pH between 3 and 5, heat resistant, and possesses low molecular weight (<1000). According its properties it isn't identical of bacteriocin.

Key words: lactobacilli, antimicrobial substance, antagonistic activity.

Dairy lactobacilli are widerely used to process yogurt and acidophilus milk. Due to the fact that viable lactobacilli can inhibit food-borne and enteric pathogenic microorganisms by producting lactic acid, hydrogen peroxide and other antimicrobial substances [5], yogurt and acidophilus milk have been considered to be healthy probiotic diet. Since Mechnikov has suggested the positive role of dairy lactobacilli for human health nearly a century ago [9], various brands of yogurt have been ingested as prophylaxis or as treatment for common intestinal infections, such as diarrhea [10], and even for vaginal bacterial [7] and yeast [4] infections. Metabolitic activation of carcinogens by the intestinal flora of humans and animals is suppressed by feeding them lactobacilli. Similarly, chemically induced colon cancer is reduced in experimental animals given lactobacilli [6]. Therefore, in addition to being nutritious and delicious food, yogurt and acidophilus milk may also promote human health by inhibiting common microbial pathogenes.

To improve the stability of dairy *Lactobacillus* starter cultures, it is important to test whether dairy *Lactobacillus* cultures possess wide antimicrobial spectrum against different pathogenes and release potent bacteriocins that may attack other *Lactobacillus* strains.

In this article we present the data about antibacterial activity of a commercial dairy *Lactobacillus johnsonii* strain designated La1.

Materials and Methods

Bacterial strain, isolation, and culture conditions. *Lactobacillus johnsonii* strain La1 was isolated from commercial probiotic yogurt Lc1 (Nestle-Mis Co.) sold at the Turkish food market. To isolate *Lacrobacillus* strain from this product, a loopfull of

yogurt was streaked on the selective MRS agar (pH 6.2) and incubated in a candle jar at 37°C for 48 h. The isolate from MRS agar was further confirmed to be a *Lactobacillus* spp. by its rod cell morphology, gram-positive stain, and catalase negative reaction. This isolate was identified by comparing its sugar fermentation patterns with the scheme described in *Bergey Manual* [7]. The control *Lactobacillus* type strains used in this study are listed in Table 1.

Table 1

Control *Lactobacillus* type strains used in this study

Strain[a]
Lactobacillus delbrueckii subsp. bulgaricus ATCC 11842
Lactobacillus delbrueckii subsp. delbrieskii ATCC 9649
Lactobacillus delbrueckii subsp. lactis ATCC 15808
Lactobacillus casei ATCC 27139
Lactobacillus acidophilus ATCC 4357
Lactobacillus acidophilus ATCC 33200

Determination of inhibitory activity by microbiological assay. Inhibitory activity was determined against various bacterial strains: *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Streptococcus group B, Candida* spp., *Helicobacter pylori, Serratia* spp., *Enterobacter cloaceae, Gardnerella vaginalis,* and various genera lactic acid bacteria of human origin using agar well-diffusion method [11]. Indicator bacteria were grown in brain-heart infusion (BHI) broth and were adjusted to 10^8 CFU/ml, and 0.1 ml of the cultures were spread onto the surface of BHI agar plates.Wells with 6 mm in diameter were cut in BHI media plates seeded with an indicator cultures using sterile tube. The cell-free culture (50 µl) extract previously adjusted to pH 7.0 and supplemented at a final concentration with 1 mg/ml of filter sterilized catalase (2600 U/mg, Sigma) to inhibit the action of hydrogen peroxide was placed into wells. After diffusion of the extract into the agar (4 h at 4°C), the agar plates were incubated overnight in a candle jar at 37°C, and the diameter of the inhibition zone around wells was measured. The final inhibition diameter corresponded to the difference between the total inhibition zone and the diameter of the well.

Termal stability. To test for heat sensitivity, the culture supernatant fluid (10 000 rpm for 15 min) was heated at 121°C for 15 min. The residual inhibitory acivity was then determined by using *E. coli* ATCC 35218 as an indicator.

pH stability. The pH of the culture supernatant fluid of La1 was raised from 4.5 to 5.3 and 6.7 with 1 N NaOH. Inhibitory activity was tested at different pHs by using *E. coli* ATCC 35218 as an indicator.

Stability to proteases. The following enzymes were tested for their hydrolytic activity: 1) the proteolytic enzymes proteinase K (2.6 U/mg), pepsin (16 milliAnson U/mg), trypsin (39 U/mg), α-chymotrypsin (45 U/mg); 2) the lipolytic enzyme lipase (900 U/mg) and 3) the glycolytic enzyme a-amylase (2.2 U/mg). All enzymes were obtained from Sigma Chemical Co., St. Louis, Mo. Assays were performed at the final concentration

[a] ATCC, American Type Culture Collection

of 1 mg/ml at pH 3.0 for pepsin, and pH 7.0 for the other enzymes. Samples with and without enzymes were held at the appropriate temperature (25°C to 37°C depending on the enzymes) for 1 h. The remaining activity after enzyme digestion was detected by the agar well-diffusion method against sensitive indicator (*Pseudomonas aeruginosa*).

Results

Inhibitory activity of *L. johnsonii* strain La1 was determined against thirteen indicator species. The zones of inhibition (diameter in millimeters) are shown in Table 2.

Table 2

Inhibitory effect of *Lactobacillus johnsonii* strain La1 on other bacteria

Bacterium (no. of strain tested)	No. of strains at the following inhibitory zone diameter (mm)						
	0	7	10	12	14	16	>16
Escherichia coli (11)			4	7			
Streptococcus gr.B (12)				8	4		
Pseudomonas spp. (2)				2			
Serratia spp. (8)				3	2	1	2
Candida spp.(18)			4	5	7	2	
Helicobacter pylori (4)			2	2			
Gardnerella vaginalis (1)				1			
Enterobacter cloaceae (2)					2		
Staphylococcus aureus (4)			3	1			
Enterococcus spp. (2)	1	1					
Lactococcus spp. (2)		2					
Pediococcus spp. (2)		1	1				
Lactobacillus spp. (9)	9						

The La1 culture supernatant was inhibitory against strains of *E coli, Streptococcus group B, Pseudomonas aeruginosa, Serratia spp., Candida spp., Helicobacter pylori, Gardnerella vaginalis, Enterobacter cloaceae* and *Staphylococcus aureus.* The cell-free supernatant of strain La1 showed also a weak activity against a few lactic acid bacteria, especially *Lactococcus* spp., *Pediococcus* spp., *Enterococcus* spp. It appeared that the strain La1 presented a broader spectrum of antimicrobial action on both gram-positive and gram-negative bacteria. But no inhibition was demonstrated against any of the nine strains of *Lactobacillus* tested.

L. johnsonii La1 excreted in the medium substances other than organic acid and hydrogen peroxide since activity was still observed after neutralization of the culture supernatant at pH 7.0 and treatment with catalase.

The antimicrobial material was active at pH between 3 and 5. When pH was raised to 5.3 the inhibitory activity was lost (Table 3). Back-titration of the substance to a lower pH allowed the inhibitory effect to be retrieved. Strain La1 retained good activity after it was heated at 121°C for 15 min. It was relatively thermostable espesially in acidic condition.

Table 3

Effect of heat on antimicrobial substance from L. johnsonii strain La1 at various pH values (the values indicate the percentage of residual activity after treatment at 121°C for 15 min)

pH 3	pH 4.5	pH 5.3	pH 6.7
100	98	47	11

Antimicrobial substance of *L. johnsonii* Lai was also stable to protease inhibitiors such as trypsin, proteinase K, α-chymotrypsin, pepsin. These characterictics indicated that the substance didn't contain a proteinaceous part in its structure. But all activity was lost after proteolytic digestion by α-amylase and lipase (Table 4). Residual activity was measured by the agar well-diffusion method. Indicator strain is given in Materials and Methods: P*seudomonas aeruginosa* ATCC 19145.

Table 4

The effect of proteolytic, glycolytic and lipolytic enzymes on the antimicrobial substance from L. johnsonii strain La1

Enzymes	Diameter of the zone of inhibition (mm) with cell-free supernatant of cultures
Catalase	12
Proteinase K	12
Pepsin	12
Trypsin	12
α-Chymotrypsin	12
α-Amylase	0
Lipase	0

Discussion

It has been reported by several investigators that lactobacilli are able to produce antimicrobial substances when grown in specific media [3]. Vincent et al. [12] described in 1959 what was called lactodicin, a substance obtained from solid agar medium seeded with *L. acidophilus.* This substance was more active against gram-negative than gram-positive bacteria.

Barefoot and Klaenhammer [2] reported a substance produced by *L. acidophilus* N2 which was active against *L. leishmannii, L. helveticus, L lactis*, and *L. bulgaricus.* It was pH dependent with maximum activity detected in broth cultures at pH 6.0. This substance was classified as a bacteriocin, because it had a molecular weight 6.500; however, its activity was restricted to members of the family *Lactobaciliaceae*, and it had a peptide structure.

Other antibacterial substances have been isolated from various types of bacteria. Microcins, for example, are produced mostly by members of the family *Enterobacteriaceae.* Microcins are peptide antibiotics with low molecular weights. They are also insensitive to proteases [1].

In contrast, bacteriocins, which are inhibitory substances that are generally produced by gram-positive bacteria, have high molecular weights and are susceptible to proteases; and their spectrum of antimicrobial activity is limited to related species [11].

The inhibitory substance produced by *Lactobacillus johnsonii* strain La1, even though it is produced by a gram-positive organism, has a low molecular weight and is active against a broad spectrum of gram-negative and gram-positive organisms, including lactic acid cocci, but not against other lactobacilli. These characteristics make the substance different from the bacteriocins. It is resistant to various proteases such as proteinase K, α-chymotrypsin, trypsin, pepsin. It is also heat resistant. The low molecular weight and its narrow range of pH preference, suggest that antibacterial substance may be a short-chain fatty acid.

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АНТИМІКРОБНА РЕЧОВИНА З ПРОМИСЛОВОГО ШТАМУ LACTOBACILLUS

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Резюме

Lactobacillus johnsonii штам La1, який був ізольований з комерційного йогурту Lc1 компанії "Нестле", виробляє речовину сильної антагоністичної активності щодо багатьох патогених та умовно-патогених мікроорганізмів і деяких молочнокислих коків, але вона не пригноблювала ріст інших лактобацил. Антимікробна речовина цього штаму активна в межах pH 3 і 5, стійка до нагрівання та має низьку молекулярну вагу (< 1000). За своєю характеристикою вона не відноситься до бактеріоцину.

Ключові слова: лактобацили,. антимікробна речовина, антагоністична активність.

АНТИМИКРОБНОЕ ВЕЩЕСТВО ПРОМЫШЛЕННОГО ШТАММА LACTOBACILLUS

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Резюме

Lactobacillus johnsonii штамм La1, изолированный из промышленного йогурта Lc1 компании "Нестле", выделяет вещество с сильной антагонистической активностью по отношению ко многим патогенным и условно-патогенным микроорганизмам и некоторым молочнокислым коккам, но не угнетает другие лактобациллы. Антимикробное вещество этого штамма активно при значениях pH от 3 до 5, термостабильно и обладает низким молекулярным весом (< 1000). По своим характеристикам оно не идентично бактериоцину.

Ключевые слова: лактобациллы, антимикробное вещество, антагонистическая активность.