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Adhesive Properties and Acid-Forming Activity of Lactobacilli and Streptococci Under Inhibitory Substances, Such as Nitrates

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Abstract One of the main requirements for probiotics is their ability to survive during passage through gastrointestinal tract and to maintain their activity at different adverse conditions. The aim of the study was to look for the strains of lactobacilli and streptococci with high adhesive properties even affected by inhibitory substances, such as nitrates (NO_3^-). To study the adhesion properties hemagglutination reaction of bacterial cells with red blood cells of different animals and humans was used. The acid formation ability of bacteria was determined by the method of titration after 7 days of incubation in the sterile milk. These properties were investigated at different concentrations of NO_3^-. The high concentration (mostly ≥2.0 %) NO_3^- inhibited the growth of both lactobacilli and streptococci, but compared with streptococcal cultures lactobacilli, especially *Lactobacillus acidophilus* Ep 317/402, have shown more stability and higher adhesive properties. In addition, the concentrations of NO_3^- of 0.5–2.0 % decreased the acid-forming activity of the strains, but even under these conditions they coagulated milk and, in comparison to control, formed low acidity in milk. Thus, the *L. acidophilus* Ep 317/402 with high adhesive properties has demonstrated a higher activity of NO_3^- transformation.

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Introduction

Nitrates (NO_3^-) and nitrites (NO_2^-) are part of human diet. Human exposure to these substances is mainly associated with three food sources: vegetables, processed meat, and drinking water [1]. They are used as traditional inhibitors of microorganism growth in processes of food fermentation. NO_3^- are also applied for protection of meat from spoilage during storage, contributed to the cured meat flavor, fixed color, and effectively controlled rancidity by inhibiting lipid oxidation [2].

NO_3^- and NO_2^- are still considered basically as undesired in food. The main objections are based on their potential to form nitrosamines with carcinogenic potential [3, 4]. Dietary uptake of NO_3^- can increase the endogenous formation of nitroso-compounds in stomach in the following way: $NO_3^- \rightarrow NO_2^- \rightarrow$ nitrosamines [5–7]. Although NO_3^- itself has no toxic properties, the bacterial reduction of NO_3^- into NO_2^- may lead to nitrous anhydrides, which in turn generate nitrosonium ions. The latter ions react with secondary amine-forming nitrosamines, many of which are cancer-inducing agents at very low concentrations [8]. About 60 kinds of nitrosamines are known, while in cheese and meat products nitrosodiethylamine, nitrosodimethylamine, xanthine, and tyramine are widely found [9, 10]. According to the latest data of World Health Organization (WHO) the daily allowable dose of NO_3^- and NO_2^- are as follows: 5 mg NO_3^- and 0.2 mg NO_2^- per 1 kg of body weight [11]. This means that a person weighting 60 kg can consume up to 250 mg NO_3^- per day without danger to his body (in terms of $NaNO_3$—up to 500 mg). Acute poisoning is usually observed in accidental ingestion of 100–500 mg NO_3^-; the amount of 800 mg NO_3^- might be fatal and 1300–1400 mg is usually fatal. Meat products may contain <2.7–945 mg NO_3^- per

kg and <0.2–6.4 mg NO_2^- per kg; dairy products may contain <3–27 mg NO_3^- per kg and <0.2–1.7 mg NO_3^- per kg. Several vegetables and fruits contain 200–2500 mg NO_3^- per kg [11]. Owing to the fact that the concentration of NO_3^- is up building in the body from different sources, such as water, fruits, vegetables etc., accumulation of these substances has brought great harm to the body.

Besides, lactic acid bacteria (LAB) including lactobacilli and streptococci are the most commonly used microorganisms for production and preservation of foods. Their occurrence in foods and feeds coupled with their long-lived use contributes to their natural acceptance, as safe for human consumption. They are frequently used as probiotics to improve some biological functions in the host organism. Through different mechanisms they are known to send signals to activate immune cells [12]. There are reports on the protection of animals and humans by LAB against diseases, such as microbial infections and cancer [13]. There are a number of studies that the fermented food products including LAB-mediated ones have a positive effect on health status in many ways [14]. And now it is possible to design and to develop starter cultures surviving in high concentration of NO_3^- and neutralizing their negative effects on macro-organisms. For these purpose LAB must have some important properties, such as antibacterial, proteolytic, and lipolytic activities as well as high adhesive properties, resistance to acid, bile tolerance, etc.

Adhesion to the intestinal mucosa is regarded as a prerequisite for colonization of the small bowel by both pathogens and the indigenous microbiota [15]. Adhesion process is necessary step for LAB enabling them to colonize environments under the conditions where they otherwise would be washed away [16]. Adhesive molecules allow cells maintaining contacts to each other and with structures in extracellular matrix. Various inhibitory substances, such as NO_3^-, different preservatives, antibiotics, and drugs, are capable to block the adhesion of bacteria to different surfaces including not only intestinal mucosa but also blood cells.

It is suggested that high intake of nitrosamines, processed meat products, salt and salted food, and, on the other side, overweight and obesity are associated with increased risk for gastric and liver cancer [17, 18]. High intake of dairy products enriched with LAB may reduce the risk for gastric disorders. This can lead to restrictions of NO_3^- and NO_2^- levels in food and drinking water [19]. The discovery of immune-stimulating probiotics [12, 20] provides a scientific basis for some of the observed probiotic effects, and consequently it is important to look for the strains of probiotic LAB with high adhesive properties [15]. They will maintain such properties even affected by harmful substances, such as NO_3^-, thus contributing to the formation of the normal intestinal microbiota [20, 21].

The aim of the present study was to find out lactobacilli and streptococci strains with high adhesive properties and to determine their activity of NO_3^- transformation.

Materials and Methods

Bacterial Cultures and Cultivation

The study was performed with LAB (125 strains) using the species of Lactobacillus acidophilus, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus jugurti*, *Lactobacillus helveticus*, *Streptococcus lactis*, and *Streptococcus thermophilus*. These LAB strains were obtained from a wide variety of dairy sources like yogurt, matsoun, cheeses and also from feces; they were identified on the basis of their morphological and biochemical characteristics according to Bergey's Manual of Determinative Bacteriology [22] in the Laboratory of Fermenting Microorganisms, Institute of Microbiology (SPC "Armbiotechnology"), National Academy of Sciences of Armenia (Yerevan). Of the 125 strains tested, 12 were found to be more active and with high adhesive properties. So, the following strains have been chosen to study: *L. acidophilus* Ep 317/402 "Narine" (INMIA[1]-9602), *L. delbrueckii* subsp. *bulgaricus* var. mazuni "Karine" (INMIA-9603), *L. jugurti* ЛІ11П (INMIA-9606), *L. helveticus* 36, *Streptococcus lactis* 1304 (INMIA-9607), *S. thermophilus* M_7 (INMIA-9608). These strains were deposited at the Microbial Depository Center of Armenia (WDM 803). Lactobacilli strains were kept in a viable condition by sub-culturing once a month in 2.5 % fat sterile milk.

The bacterial strains were grown in 2.5 % fat sterile milk supplemented with 0.5–2 % NO_3^- ($NaNO_3$ and KNO_3 were used). Growth medium was inoculated with overnight cultures of LAB and incubated at 37 °C. Then these cultures were plated onto solid nutrient medium (MRS agar, HiMedia, Mumbai, India) [14, 23] in Petri dishes and incubated at 37 °C for 24–48 h. Afterwards isolated pure colonies of LAB were used in hemagglutination reaction with red blood cells (erythrocytes)

Preparation of Erythrocyte Suspensions and Adhesion Assays

To study the adhesion properties of bacterial cells, hemagglutination reaction with erythrocytes of different animals and humans is widely used: hemagglutination with at least one of those red blood cells indicates the presence of bacterial adhesion factors [24–26]. The assay with preparing erythrocytes in formalin was carried out as

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follows: an appropriate quantity of blood (human, sheep, bull) was defibrinized and filtrated as a final concentration of 8 % erythrocytes. Then 3 % formalin solution was prepared in potassium–phosphate buffer (pH 7.2) and to this solution was added the 8 % suspension of erythrocytes. The medium was incubated at 37 °C for 24 h. After incubation the suspension was centrifuged three times at 2500 g and washed in phosphate-buffered saline thrice (1.2 g L^{-1} Na_2HPO_4; 0.4 g L^{-1} KH_2PO_4; 9.0 g L^{-1} NaCl). After, sediment was reached of the original volume of fresh buffer and 40 % formalin solution was added, as a final concentration of 0.5 % erythrocytes. The adhesion of LAB on erythrocytes was studied on glass substrates.

Determination of Acid-Forming Activity

The acid formation ability was determined by the method of titration [27]. The stored amounts of LAB in a minimum infection of sterilized milk for 17 h (acid-forming energy) and during 7 days (limiting acidity) were determined. 20 ml of distilled water and one or two drops of indicator phenolphthalein were added to 10 ml sterilized milk and kept at 37 °C. Titration was performed with 0.1 mol L^{-1} NaOH until light pink color.

Results were expressed in Terner degrees (^0T) and counting by formula: $K = 10\times$; where K is degree of acid formation, X—amount of NaOH in ml used in titration, 10—coefficient of transferring ml into ^0T.

Data Processing

The average data processed were statistical of three replicates of independent measurements. The SigmaPlot software was used to determine the standard errors (<5 %). Data were subjected to statistical analysis with reliability criteria of Student's t test (Microsoft Excel 2010). Differences between experimental and control data were considered as statistically valid when $p < 0.05$ [23].

Results and Discussion

Effects of NO_3^- on Lactobacilli and Streptococci Survival

In our research, we have started the study with 500–600 mg (0.5–0.6 %) concentration of NO_3^-. Hence our LAB species and strains were resistant against these concentrations, so we decided to increase the concentrations up to 2000 mg (2 %) and thus to reveal the properties of LAB in hazardous conditions. The investigation of NO_3^- influence on the growth of LAB especially lactobacilli and streptococci has shown that the high concentrations of NO_3^- (mostly ≥2.0 %) inhibited bacterial growth of the most strains studied but *L. acidophilus* Ep 317/402, *L. acidophilus* 5e, or *L. acidophilus* EA_2 strains were able to grow in the presence of NO_3^- (Table 1). Moreover, some streptococci strains namely *S. lactis* 1304 and *S. thermophilus* M_7 did not grow in the presence of NO_3^- in the concentrations of ≤1 % (see Table 1). In spite of the inhibitory effect of NO_3^- on bacterial growth, the probiotic LAB sometime coagulated the milk, but these starters couldn't coagulate the milk after several inoculations.

Adhesion ability of Lactic Acid Bacteria

The adhesion ability of different lactobacilli and streptococci strains was determined using hemagglutination reaction with red blood cells of human, sheep and bull (Table 2); the strains survived at different concentrations of NO_3^-. In the case, when the concentrations of $NaNO_3$ and KNO_3 reached 2–2.5 %, the most of strains were unable to grow and consequently showed no adhesion activity (Fig. 1). Interestingly, the streptococcal cultures, especially *S. lactis* 1304 and *S. thermophilus* M_7, had not shown ability to survive at high concentrations of NO_3^- (see Table 1) but they revealed adhesion properties (see Fig. 1). Moreover, some lactobacilli strains namely *L. acidophilus* Ep 317/402, *L. acidophilus* 5e, or *L. acidophilus* EA_2 demonstrated the highest adhesive properties (see Fig. 1).

The reaction with erythrocytes of different animals significantly enhances the ability to identify adherent bacteria [25]. On this basis, to identify the adhesive properties of LAB erythrocytes of different animals have been used. The most of strains, especially *L. acidophilus* Ep 317/402, *L. acidophilus* 5e, and *L. acidophilus* EA_2 caused evident hemagglutination of human erythrocytes compared with bovine and sheep ones (see Fig. 1). Only *S. thermophilus* M_7 didn't cause any reaction with human erythrocytes.

The adhesive properties worsened directly proportional to the concentration of NO_3^-. Reaching the concentration to 2 % (fourfold increase) led to decrease of adhesion ($p < 0.05$) (see Fig. 1). Moreover, in cases of bovine and sheep erythrocytes no one demonstrated adhesion ($p < 0.01$). In addition, it is known that the symptoms of NO_3^- and NO_2^- poisoning of sheep are decreasing hemoglobin quantity and increasing methemoglobin content of blood [28]. Interestingly, it was shown that in Gram-positive bacteria, such as streptococci, the existence of pili, formed by covalent polymerization of adhesive pilin subunits, suggested the ability of streptococci to adhere and to attach to host cells [7].

The strength of adhesion of probiotic cells to epithelial cells is critical for the probiotics effect against pathogenic

Table 1 Growth of lactobacilli and streptococci at the presence of different concentrations of NO_3^-

LAB species ad strains	Concentrations of NO_3^- (%)							
	$NaNO_3$				KNO_3			
	0.5	1.0	1.5	2.0	0.5	1.0	1.5	2.0
L. acidophilus 3e	+	+	+	−*	+	+	–	–
L. acidophilus 5e	+	+	+	+	+	+	+	–
L. acidophilus 8e	+	+	+	–	+	+	+	–
L. acidophilus EH_1	+	+	–	–	+	–	–	–
L. acidophilus EH_2	+	+	+	–	+	+	–	–
L. acidophilus EA_2	+	+	–	–	+	+	+	+
L.acidophilus 317/402 "Narine"	+	+	+	+	+	+	+	–
L. delbrueckii subsp. *bulgaricus* var. *mazuni* "Karine"	+	+	+	–	+	+	–	–
L. helveticus 36	+	+	–	–	+	+	–	–
L. jugurti ЛІІІП	+	–	–	–	+	+	+	–
S. thermophilus M_7	+	+	–	–	+	+	–	–
S. lactis 1304	+	+	–	–	+	–	–	–

*(−) Means the absence of growth

Table 2 Adhesion ability of LAB on red blood cells of different animals and human under certain culture conditions

LAB species and strains	Red blood cells		
	Human	Bovine	Sheep
L. acidophilus 3e	+	+	+
L. acidophilus 5e	+	+	+
L. acidophilus 8e	+	+	–
L. acidophilus EH_1	+	+	+
L. acidophilus EH_2	+	+	+
L. acidophilus EA_2	+	+	+
L. acidophilus 307/412"Narine"	+	+	+
L. delbrueckii subsp. *bulgaricus* var. *mazuni* "Karine"	+	+	+
L. helveticus 36	+	+	+
L. jugurti ЛІІІП	+	–	+
S. thermophilus M_7	+	+	+
S.lactis 1304	+	+	+

*(−) Means the absence of adhesion

bacteria [15]. The adhesion capacity of the probiotic bacteria was determined in cultures of different epithelial cells line, as Caco-2 or Hela cells [29–31], but there were insufficient data concerning to erythrocytes. Moreover, as it was mentioned [32], the benefits of probiotics against cancer are attributed to competitive adhesion of pathogenic bacteria, direct physical binding to carcinogens, altering intestinal environment to modulate the production of enzymes, antioxidant activity, and immune modulation.

Adhesion ability has been suggested to be an important property of many bacterial strains used as probiotics, which play an important role in the formation of biofilms to protect the hosts from colonization by pathogens [33]. The results obtained allow to conclude that during bacterial fermentation NO_3^- were accumulated in the milk, transformed into NO_2^- and then to nitrosonium ions generating nitrosamines, as suggested [5–7]. And these substances even in the low concentrations might cause structural and metabolic changes in bacteria; further sub-culturing harmful effect was detected: LAB lost their viability.

Different adhesion properties of lactobacilli and streptococci may be considered with adhesins of colonization factor antigens (CFA) type. The most characteristic among CFAs are CFA I, CFA II and CFA IV (also known as PCF8775 fimbriae). These fimbriae antigens contained several epitopes; it was shown that *L. acidophilus* 317/402 "Narine" had CFA I adhesins and *S. thermophilus* M_7—adhesins like P fimbriae [25].

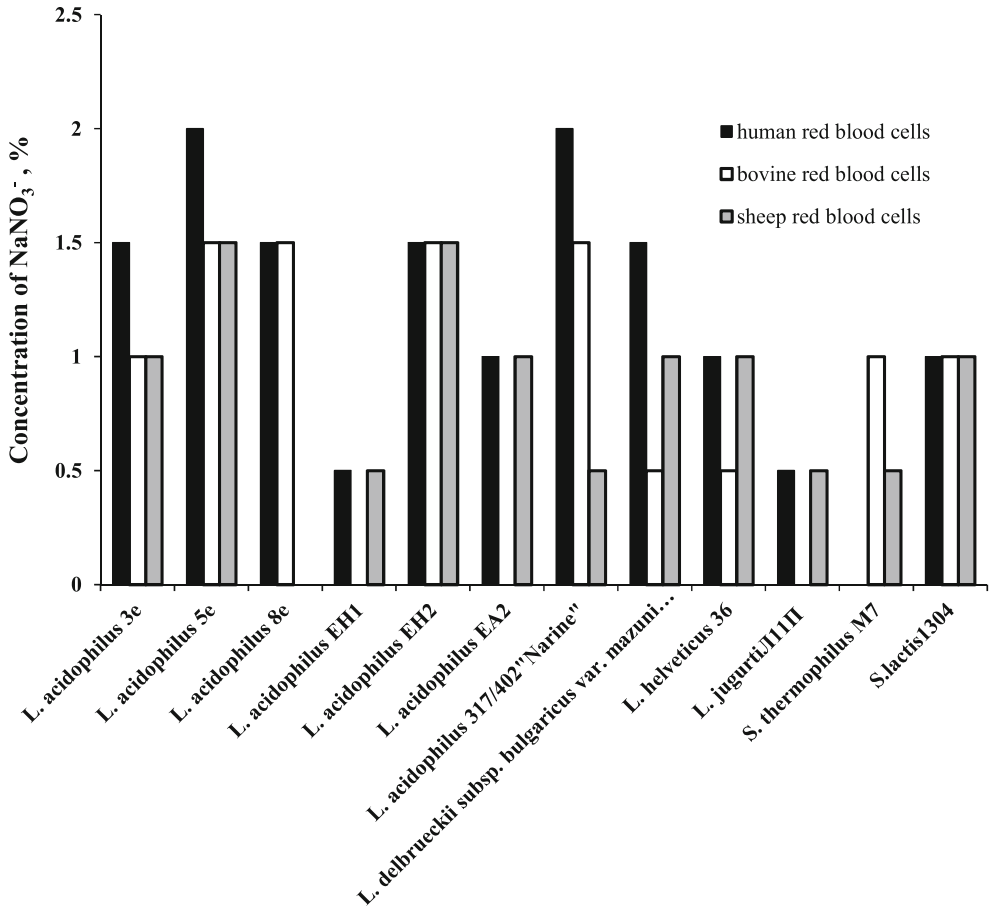

Fig. 1 Adhesion properties of lactobacilli and streptococci at different concentrations of $NaNO_3$ evaluated by hemagglutination reaction with human (*filled black square box*), bovine (*open white square box*) and sheep (*filled gray square box*) red blood cells

Assessment of Acid-Forming Activity by Lactobacilli and Spreptococci

The major principle involved in food and feed preservation by probiotic LAB is their ability to survive during passage through the gastrointestinal tract, to adhere and to colonize the intestinal epithelial cells and to produce lactic acid, which exerts most of the inhibitory capacity against pathogenic microorganisms. It was revealed that when lactic acid is produced, the pH decreases. Probiotics ferment undigested carbohydrate residues preferentially in the proximal colon resulting in high levels of short chain fatty acids (SCFAs). Undigested protein residues from the gut are also fermented more distally in the colon and produce branched SCFAs, hydrogen sulfide, ammonia, and several phenolic and indolic compounds [34]. The major SCFAs produced are acetate, propionate, and butyrate. It has been shown that non-dissociated SFA and their penetration through bacterial membranes cause weak acid anions, which are accumulated in cytoplasm, thereby affecting on metabolic processes [35]. Growth inhibition occurs and the microorganisms eventually die. Additionally butyrate is an important tumor suppressant molecule [36].

The results of acid-forming activity by LAB at different concentrations of NO_3^- (0.5–2.0 %) were recorded on the seventh day (Table 3). The pH of medium was observed to decrease during lactobacilli growth, as shown before [32]. This might be explained due to that, within 7 days, organic acids in milk continued to decompose. The high concentration of acid components in milk had the inhibitory effect on cell viability, and the process of cellular breakdown began due to the lack of nutrients. The cell density decreased and, in addition, the presence of high concentration of inhibitors affected the acid-forming process (see Table 3).

It is known that the cells which grow in acidic conditions usually acquire acid tolerance by accumulating acid stress proteins inside the cell [23, 37]. This could be useful for investigation of bacterial maximal biomass yield, survival ability under the influence of inhibitory substances, and at different pH values on adhesive properties that can be used for industrial applications of LAB in dairy milk

Table 3 The effects of different concentrations of NO_3^- on acidity production by lactobacilli and streptococci

LAB species and strains	Acidity (°T)							
	Control (without NO_3^-)	Concentrations of NO_3^- in milk (%)							
		$NaNO_3$				KNO_3			
		0.5	1.0	1.5	2.0	0.5	1.0	1.5	2.0
L. acidophilus 3e	350 ± 0.1	290 ± 0.6	170 ± 0.6	135 ± 0.6	−*	277 ± 0.3	110 ± 0.3	–	–
L. acidophilus 5e	330 ± 0.3	260 ± 0.3	130 ± 0.3	90 ± 0.3	61 ± 0.1	240 ± 0.3	110 ± 0.3	63 ± 0.3	–
L. acidophilus 8e	320 ± 0.3	230 ± 0.1	120 ± 0.2	80 ± 0.3	–	220 ± 0.6	105 ± 0.1	75 ± 0.3	–
L. acidophilus EH_1	325 ± 0.3	290 ± 0.3	130 ± 0.3	–	–	267 ± 0.3	–	–	–
L. acidophilus EH_2	335 ± 0.6	265 ± 0.6	140 ± 0.1	72 ± 0.6	–	230 ± 0.2	110 ± 0.1	–	–
L. acidophilus EA_2	335 ± 0.3	260 ± 0.3	155 ± 0.3	–	–	220 ± 0.1	105 ± 0.1	72 ± 0.3	45 ± 0.1
L. acidophilus 317/402 "Narine"	340 ± 0.3	270 ± 0.3	150 ± 0.3	120 ± 0.3	80 ± 0.1	250 ± 0.3	99 ± 0.1	62 ± 0.6	–
L. delbrueckii subsp. *bulgaricus* var. *mazuni* "Karine"	290 ± 0.1	165 ± 0.3	66 ± 0.3	58 ± 0.3	–	147 ± 0.6	68 ± 0.3	–	–
L. helveticus 36	300 ± 0.3	200 ± 0.3	60 ± 0.3	–	–	185 ± 0.2	80 ± 0.1	–	–
L. Jugurti $_{\text{J1 1П}}$	280 ± 0.6	190 ± 0.2	–	–	–	155 ± 0.3	70 ± 0.1	60 ± 0.3	–
S. thermophilus M_7	130 ± 0.3	88 ± 0.3	64 ± 0.3	–	–	88 ± 0.3	66 ± 0.2	–	–
S. lactis 1304	120 ± 0.3	72 ± 0.3	54 ± 0.6	–		95 ± 0.3	–	–	–

*(−) Means the absence of growth

product technology as starters like probiotics and in medicine.

Conclusions

The results suggest that the high concentration, mostly ≥2.0 % of NO_3^- inhibited the growth of both kind of LAB species (lactobacilli and streptococci), but compared with streptococcal cultures lactobacilli have shown more stability and high adhesive properties. Among them *Lactobacillus acidophilus* Ep 317/402 demonstrated more stability and a higher adhesive property.

Further research will be performed to evaluate remaining attributes related to adhesion.

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Compliance with Ethical Standards

Conflict of interest There is no conflict of interest.

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