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HEALTH PROMOTING POTENTIALS OF ARMENIAN FUNCTIONAL SOUR MILK "NARINE" AND ITS STARTER LACTOBACILLUS HELVETICUS MDC 9602

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Abstract

This investigation was undertaken for in vitro evaluation of the adaptive and probiotic properties of Lactobacillus helveticus MDC 9602, the starter of the Armenian functional sour milk "Narine" widely used for curing gastrointestinal disorders and lowering blood pressure. Lactobacillus helveticus species predominantly are used as starters for production of cheeses and fermented sour milk drinks, but a growing body of scientific evidence shows that some strains belonging to this species possesses health benefits. The adaptive properties of MDC 9602, necessary for bacteria viability in the gastrointestinal tract, involve high tolerance to acids, high auto aggregation and hydrophobicity, but low tolerance to bile (0.3%) and NaCl (2%); it does not hydrolyze bile salts, nor utilizes maltose. L. helveticus MDC 9602 produces antimicrobials sensitive to proteinase K and large amounts of lactic acid, exhibits an antagonistic effect on pathogenic and related bacteria. It is susceptible to many routinely prescribed antibiotics and can considered safe.

It is susceptible to many routinely prescribed antibiotics. The beverage "Narine" besides of $>10^{11}$ CFU/g friendly bacteria, contains a high amount of different proteinases and β-galactosidase, which increases nutritional bioavailability of food. Taking "Narine" prevents gastrointestinal infections, modulates the host's immune response, improves the composition of the gut microbiota and lowering high blood pressure. Most of the listed properties of L. helveticus MDC 9602 do not appear in media other than milk. Thus, it was possible to show that the fermented milk product "Narine", along with live friendly bacteria, also contains a high amount of various hydrolases, lactic acid and bioactive molecules that improve human health and its starter L. helveticus MDC 9602 can be included among the bacterial species commonly considered as probiotics and starter cultures for the production of high quality nutraceuticals.

Keywords: *Lactobacillus helveticus, dairy starter, sour milk "Narine", probiotic, health benefits.*

Introduction

Fermented sour milk beverage "Narine" patented by Professor Levon Yerzinkyan in 1964 as functional food promoting human health, for more than six decades has been widely used as a natural

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preparation to cure different kinds of gut disorders. The author erroneously attributed starter of "Narine" to the species of *L.acidophilus*, despite more than a dozen morphological, biochemical and cultural differences from typical strains of *L. acidophilus* and incapability to ferment milk [*Kvasnikova E et al., 1967; Erzinkyan L, 1971*].

According to polymerase chain reaction amplification and sequencing of 16S rRNA gene fragments (1 - 1359 bp), RNA polymerase rpoA gene (1 - 780 bp) [*NCBI GenBank under numbers HQ379170 and HQ379179, Pashayan MM, Hovhannisyan HG, 2010*] and a battery of morphological, physiological and biochemical tests, the starter of "Narine" has been re-identified as *Lactobacillus helveticus* MDC 9602.

L. helveticus is an obligate homofermentative lactic acid bacterium (LAB), which is widely used as a starter culture to manufacture yogurt and certain Swiss and Italian cheeses. Milk fermented with certain *L. helveticus* strains has been shown to become enriched with antihypertensive and immunomodulatory bioactive peptides [*Griffiths M, Tellez A, 2013*]. Although *L. helveticus* is commonly associated with milk environments, this species has also been recovered from whisky fermentations and more sporadically, from the human vagina and gastrointestinal tract [*Broadbent J et al., 2011*].

The species of *L. helveticus* is phylogenetically very closely related to *L. acidophilus* (the 16S rRNA gene sequences of these bacteria differ by only 1.6-1.7% [*Callanan M et al., 2008*; *Hovhannisyan H et al., 2020*]. However, while *L. helveticus* is a specialized dairy starter, *L. acidophilus* is a natural inhabitant of the human gut and hardly grows and ferments milk. Many strains of *L. acidophilus* are widely used as probiotics, but a growing body of scientific evidence shows that some strains belonging to the *L. helveticus* species also have health benefits. According to the World Health Organization, probiotics are "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host". The probiotic microorganism should be bile-tolerant, acid-resistant, non-pathogenic, non-carcinogenic, adhere to host epithelial tissue, enrich the intestinal microflora, hinder pathogenic adherence, and possess antimicrobial activity [*Pan X et al., 2009*; *Prabhurajeshwar C, Chandrakanth R, 2017*].

Many probiotic bacteria produce a wide range of effective antimicrobials, including lactic acid, hydrogen peroxide, and bacteriocins, which are often used to control the growth of pathogens in fermented foods. Unfortunately, the probiotic properties of *L. helveticus* and the functionality of the "Narine" remain poorly understood.

The purpose of this study is to evaluate the functional and probiotic features of fermented milk "Narine" and its starter culture *L. helveticus* MDC 9602.

Materials and methods

L. helveticus MDC-9602 (*LAB-1)*, *L. helveticus* ATCC 15009 (American Type Culture Collection) (*LAB-2)*, and *L. acidophilus* NCFM (North Carolina Food Microbiology) (*LAB-3)*, were obtained from the Microbial Depository Center (MDC) of the SPC "Armbiotechnology" of the National Academy of Sciences of the Republic of Armenia. Skimmed milk, hydrolyzed milk, MRS (De Man, Rogosa and Sharpe agar) and LAPTg (5% peptone, 1% tryptone, 1% glucose, 1% yeast extract, 0.1% Tween 80, pH 6.5) were used as culture media [*Hovhannisyan H et al.,2020*] (Taurodeoxycholic acid sodium salt Sigma, USA).

Utilization of sugars**:** overnight cultures of LAB 10^5 *CFU/ml* were plated on LAPTg agar containing the appropriate sugars as the main source and incubated at 37°C. The growth was assessed the next day.

Proteolytic activity assay**:** overnight cultures were precipitated by centrifugation, the supernatant was poured into a sterile test tube, paper discs were immersed in the supernatant and placed on a milk-agar medium.

Titratable acidy assay**:** to 10 *mL* of fermented milk was added 3 drops of phenolphthalein as an indicator, then the mixture was titrated using 0.1 N NaOH to form a pink color. The total value of titratable acid was calculated by converting it to lactic acid percentage.

Antioxidant activity assay: was performed by 1.1-diphenyl-2-picrylhydrazyl radical inhibition [*Apostolidis E et al., 2006*].

Antibiotic susceptibility assay**:** carried out by disk-diffusion method. LAB cultures 10^6 *CFU/mL* were seeded on MRS agar, disks with antibiotics were placed on a bacterial lawn, and the diameter of the zone of inhibition by each antibiotic was determined after incubation at 37°C for 24 hours.

Bacterial hydrophobicity assay: was determined by microbial adherence to solvents with slight modifications [*Bellon-Fontaine M et al., 1996*]. Cells harvested by centrifugation were washed and suspended with phosphate-buffered saline buffer at pH 7.1 to concentration of 10^8 *CFU/ml*, then 5 *ml* was mixed with 1 *ml* of xilene. Following incubation at room temperature for 10 *min*, the mixture was vortexed vigorously for 2 *min* and then allowed to stand for 15 *min* to ensure complete separation of the organic and aqueous phases. The absorbance of the aqueous layer was measured at 600 *nm*.

The adhesion coefficient of bacteria to solvents was calculated as follows.

$$\text{Adhesion} = (1-\frac{A}{A_o})\times 100$$

Where optical density A was measured within 1-5 hours and the optical density A_0 after 0 hours.

Bacterial autoaggregation assay: Cultured bacteria harvested by centrifugation were washed twice with phosphate-buffered saline to reach absorbance value of 0.3 ± 0.05 at 600 *nm*. To determine autoaggregation percentage, bacterial suspension was incubated at 37°C for 4 and 24 *h*. Absorbance at 600 *nm* was measured at 0, 4, and 24 *h* after incubation. The percentage of autoaggregation was expressed as follows [*Collins J et al., 1998*].

$$\text{Autoaggregation} = (1-\frac{A_t}{A_o})\times 100$$

Where A_0 and A_t represented the absorbance at 0 *h* and at indicated incubation time (4 and 24 *h*), respectively.

Bile and acid sensitivity assay: Bile and acid sensitivity assay: were performed by known methods [*Hyronimus B, 2000*].

Qualitative analysis of bile salt hydrolases: a colony of lactic acid bacteria was grown on LAPTg agar containing 0.5% (w/v) taurodeoxycholic acid sodium salt and 0.037% calcium chloride, and incubated under anaerobic conditions at 37°C for 72 hours. The precipitation zone surrounding the colony indicated the activity of bacterial bile salt hydrolase.

NaCl tolerance assay: In test tubes containing 5 ml of MRS broth with different concentrations of NaCl, 0.05 *ml* of an overnight culture of lactic acid bacteria was added and incubated for 24 h at 37°C. Bacterial growth was assessed using a spectrophotometer at a wavelength of 600 *nm*. Growth serves as a positive control and no growth serves as a negative control.

Statistical analysis

All experiments were performed in 3-5 repetitions. Statistical analysis of the obtained data was carried out using Student's computer test, taking the $p < 0.05$ criterion as sufficient to assess a significant difference in the results.

RESULTS

Table 1 presents the physiological, biochemical, and cultural characteristics of LAB-1 in comparison with those of LAB-3 and LAB-2strains.

The LAB-1strain ferments milk quickly, forming a clot with a very viscous consistency whereas LAB-2ferments milk more slowly without forming ropy texture andLAB-3does not ferment milk at all. Since *L. acidophilus* is adapted to body temperature, the optimum temperature for its growth is lower than that of lactic starters.

The main habitat of *L. helveticus* strains is milk but for *L. acidophilus* is gastrointestinal tract. All tested stains produced bacteriocins, which can differ each of other. In a similar trend, the hydrophobicity values obtained for LAB-1in the presence of xylene were significantly higher than for the other two strains (Table 2). The autoaggregation ability of *L. helveticus* was also significantly higher than *L. acidophilus*. The autoaggregating phenotype of LAB-1was so strong that over 50% bacteria have formed a precipitate in 1-2 *h*, while *L. acidophilus*

TABLE 1

Physiological-biochemical and cultural features of LAB-1, LAB-3 and LAB-2

Characteristic	LAB-1	LAB-3	LAB-2
Milk fermentation(h)	4 - 5	-	8 -10
Fermented milk ropiness	+++	-	±
Exoprotease activity	+++	-	+
β-galactosidase activity	+++	+	++
Auxotrophy (amino acid)	14	14	14
Optimal growth temperature (°C)	40 - 45	37-40	40 - 45

TABLE 2

Probiotic characteristics of
LAB -1, LAB -2 and LAB-3

Characteristics	LAB-1	LAB -3	LAB -2
The main habitat	milk	gut	milk
Antimicrobial production	+++	+++	++
Auto-aggregation (%)	79.5±1.4	42.3±1.2	72.8±1.6
Hydrophobicity (%)	56.8±1.0	33.8±2.1	53.4±1.6
Maltose utilization	-	+++	-
Angiotensin converting enzyme inhibitors formation (ACE)	+++	-	±
Hydrolysis of bile salts	-	++	-
Resistance to acid stress	R	S	S
Bile resistance, %	0.3	0.6	0.3
Resistence to NaCl, %	≥ 2	≥2	≥2
Antioxidant activity	58	36	42
Antibiotic resistance	-	-	-

suspension showed constant turbidity with some precipitate.

It was revealed that the starter of “Narine” (LAB-1) is highly resistant (<3.5 pH) to acid stress and outperforms to LAB-3 and LAB-2. The resistance to bile (0.3%) and NaCl (2%) is about the same for all.

Sugar metabolism is the main criterion for the typing of LABs [*Ludwing W et al., 2009*]. Comparative study of sugar metabolism revealed a significant difference between LAB-1, LAB-2 and LAB-3 (Table 2). As can be seen from the data in table 2, both lactic starters of *L. helveticus*, in contrast to the intestinal strain of *L. acidophilus*, do not use maltose, which is usually found in the intestine as a by-product of starch metabolism.

Antibiotic resistance assessment revealed that LAB-1is sensitivity of most of the tested antibiotics: kanamycin, streptomycin, rifampicin, neomycin, tetracycline, erythromycin, gentamicin, clindamycin and chloramphenicol, which indicates on its use safety.

Many of the health benefits caused by *L. helveticus* are highly dependent on the characteristics of the environment in which it grows. Therefore, a comparative study of the results of the metabolic activity of *L. helveticus* in milk and MRS was carried out (Table 3).

The data presented in table 3 shows that many of the main features of LAB-1; ropiness, viscosity, biosynthesis of ACE inhibitors, immunomodulators, bioactive peptides, extracellular proteases and β-galactosidase, expressed only in milk. The strain LAB-1 forms the highest titratable acidity in fermented milk among lactobacilli.

DISCUSSION

Bioinformatics analysis has demonstrated that although *L. helveticus* and *L. acidophilus* genomes share 98.4% identity, despite the fact that they have adapted to grow in very different environments - dairy products and the human gut, respectively. This suggests that the difference between dairy and intestinal LAB strains is determined by a relatively small but highly specific set of genes [*Callanan M et al., 2008*]. In addition, the comparative genomics of the gastrointestinal tract-associated species of *L. acidophilus*, *L. gasseri* and *L. johnsonii* with the dairy species of *L. bulgaricus* and *L. helveticus* revealed the key sets of genes that determine adaptation to a particular niche [*Makarova K et al., 2007; Callanan M et al., 2008*; *O’Sullivan O et al., 2009*].Thus, the gene for bile salt hydrolase, which is required for survival in the gastrointestinal tract, is active in *L. acidophilus* but is not functional in *L. helveticus* due to a frameshift mutation.

The type of sugar available is an important environmental factor for bacterial niche specialization [*Slattery L et al., 2010*]. The presence of the

TABLE 3

Characterization of dairy and MRS 2- day cultures of LAB-1

Characteristic	Fermented milk	MRS culture
Bacteria*(CFU/ml)* *	$6.7 \pm 1.8 \times 10^{11}$	$1.2 \pm 0.7 \times 10^{10}$
Ropiness(*cm*)	>50	0
Viscosity (*P·s*) *	118 ± 2.6	10 ± 0.3
Acidity(*pH*)	4.5 ± 0.3	5.7 ± 0.2
Titrable acidity (°*T*)	252 ± 3.7	61 ± 1.4
ACE inhibitors	+++	-
Bacteriocins	+++	+++
Immunomodulators	+++	-
Other bioactive peptides	+++	-
Extracellular proteases	+++	-
β-galactosidase	+++	-

NOTES: ** – CFU-colony-forming unit,*
P·s – Pascal second

enzyme maltose-6-phosphate glycosidase, as well as multiple copies of glucosidase genes, can be putative indicators of a gut-adapted microorganism [*Slattery L et al., 2010*]. Maltose-6-phosphate glycosidase is found solely in gut organisms and is absent even in a multi-niche organism. Adaptation to intestinal poverty is highly dependent on the number of active glycosidase genes responsible for the utilization of maltose. An analysis of all sequenced LAB genomes showed that the genes for Maltose-6-phosphate glycosidase are predominantly present in commensal intestinal microorganisms in the species *L. acidophilus, L. jhonsoni, L. casei, Enterococcus faecalis, E. faecium*and *Streptococcus suis* [*O'Sullivan O et al., 2017*].

L. helveticus species are the most nutritionally fastidious among LAB as they are auxotroph of 14 amino acids [*Christiansen J et al., 2008*]. In order to assure its nutritional requirements when grown in milk, LAB must have high proteolytic activity in order to release enough peptides and amino acids for growth. Among LAB, *L. helveticus* is characterized by rapid growth in milk due to its high proteolytic activity and resistance to acid stress [*Yamamoto N et al., 1994*]. Because of the highest proteolytic activity of the starter the titrable acidity of "Narine" can reach up to 250°T for three days of incubation. The proteolytic system of *L. helveticus* and the majority of LAB consist of exoproteinases, which initially cleave caseins to short peptides and intracellular peptidases, which further degrade these peptides to oligopeptides and amino acids. Most LAB have only one type of extracellular proteinase, while in some of *L. helveticus* strains up to four proteinases have been identified [*Kunji E et al., 1996*]. The high rate of fermentation of "Narine" is due to a very high protease activity of its starter. For rapid growth in milk, there should be equilibrium between protease and β-galactosidase activities of starter culture (Table 1). When LAB grow in milk the genes coding of proteases and β-galactosidase activated however, when they grow in peptide-rich medium with glucose (MRS) these genes are repressed.

Milk proteins have been identified as an important source of bioactive peptides. Such peptides can be released during fermentation due to hydrolysis of microbial enzymes. The peptides derived from milk proteins have been shown to have a variety of different functions in *in vitro* and *in vivo*, e.g., antihypertensive, opioid, antimicrobial, antithrombotic, immunomodulatory or mineral-carrying.

L. helveticus, thanks to a highly efficient proteolytic system consisting of a unique set of peptidases and proteinases, is able to hydrolyze milk proteins into discrete bioactive peptides, namely the tripeptides Ile-Pro-Pro and Val-Pro-Pro which as has been shown, inhibit the action of the ACE responsible for increasing blood pressure. However, not all *L. helveticus* strains are capable of producing ACE-inhibiting oligopeptides during milk fermentation [*Yamamoto N et al., 1994; Giraffa G et al., 2000*; *Seppo L et al., 2003*].

Bacteriocins are proteinaceous substances exhibiting bactericidal activity against related bacteria competing for the same resources or ecological niche. Two types of bacteriocin produced by *L. helveticus* are known – Helveticin J and Helveticin V-1829, which have a narrow spectrum that inhibits growth of a small number of Lactobacillus species. The both are large heat-labile bacteriocins, and classified as a class III bacteriocin [*JoergerM et al., 1981*] According to our data, the antibacterial activity of the cell-free supernatant of *L. helveticus* MDC 9602 is also sensitive to a temperature of 50°C and to protease K.

The health benefits of "Narine" and other dairy products fermented by *L. helveticus* are shown in table 3. Their main positive effect, demonstrated to date, is the production of ACE-inhibiting peptides. In clinical trials, these peptides have been confirmed to lower blood pressure [*Jauhiainen T et al., 2005*]. Dairy products, such as Calpis and Evolus are already on the market fermented by straines of *L. helveticus*, which has a very high identity with the "Narine" starter and *Saccharomyces cerevi*siae, contains both of known ACE-inhibitory peptides. The action of these antihypertensive peptides has been shown to decrease the systolic blood pressure in hypertensive rats after 4 to 8 *h* of consumption of Calpis sour milk [*Nakamura Y et al., 1995*]. The effect of Evolus, another L. helveticus LBK-16 H fermented milk produced in Finland, has also been demonstrated in several studies to lower blood pressure in hypertensive subjects [*Jauhiainen T et al., 2005*].

It has been demonstrated that consumption of *L. helveticus*-fermented milk results in increased cal-

TABLE 4

The health benefits of Narine and other dairy products fermented by L. helveticus

Human studies	
	Promotes overall gut health
	Treats gut infections
	Decreases blood pressure
	Improves anxiety and depression
	Improves sleep
	Mitigates respiratory tract illnesses
	Combats food allergens
	Increases calcium levels
	Has a positive effect on calcium metabolism
	Immunostimulation and immunomodulation
Mice studies	
	Learning and memory
	Arthritis
	Dermatitis
	vulvovaginal candidiasis
	Breast tumors
	Infection
	Radio protective properties studies
***in vitro* studies**	
	Cancer
	Inflammation

cium absorption compared with ordinary sour milk. *L. helveticus*-fermented milk increases bone mineral density and bone mineral content in relation to body weight in long-term feeding of growing rats [*Narva M et al., 2004*]. In another study, animal feeding trials using rats demonstrated that consumption of cheese produced with the use of *L. helveticus* resulted in the suppression of abdominal adipose tissue accumulation compared with rats given an isocaloric feed prepared using butter oil and casein. The cheese diet led to a 1.5-fold reduction in the production of adiponectin from abdominal adipose tissue [*Narva M et al., 2004*]. Daily consumption of *L. helveticus* LBK-16H fermented milk containing bioactive peptides has a proven blood pressure-lowering effect in hypertensive subjects and thus is a potential dietary treatment of hypertension [*Jauhiainen T et al.,2005*]. It is revealed that "Bon-Narine" (the Japanese trade mark of product Narine) possesses immunomodulating and immunostimulating properties, remotes phagocytic activity in the reticuloendothelial system and has a stimulatory effect on peritoneal macrophages [*Sugiura H et al., 1994*]. Oral administration of "Narine" restored the dysfunction of the microbiota of irradiated rats within one to two weeks [*Afrikian E, 2012*].

It has been established that the thermal destruction of *L. helveticus* in fermented milk does not affect many of the useful properties of the product, thus confirming the participation in the protective processes of not only probiotic bacteria, but also bioactive metabolites formed during milk fermentation [*Vinderola G et al., 2011*]. Many mainstream brands (like Dannon and Chobani, etc.) add *Lactobacillus helveticus* in their drinkable or traditional yogurts in order to promote gastrointestinal functionality.

CONCLUSION

Although *L. helveticus* MDC 9602 is not an intestinal microbe, it has health-improving properties through inhibition of pathogens, modification of gut microbiota, modulation of the host immune system, generation of bioactive peptides from food molecules, degradation of allergens that are not inferior to those of well-known probiotic microorganisms and can be included among the bacterial species commonly considered as probiotics.

Sour milk "Narine" fermented by *L. helveticus* MDC 9602, in addition to more than 10^{11} *CFU/g* of live friendly bacteria, contains a large amount of freshly synthesized proteinases, β-galactosidase, bacteriocins, lactic acid, polysaccharides and other metabolites that can treat any gastrointestinal disorders and in terms of bioactive molecules is unique among functional dairy products.

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