Section 1. Biotechnology

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Karapetyan Kristina,
Probiotics obtaining Sector,
Scientific and Production Center "Armbiotechnology" NSPO,
National Academy of Science, Yerevan, Republic of Armenia

Tkhruni Flora,
Probiotics obtaining Sector,
Scientific and Production Center "Armbiotechnology" NSPO,
National Academy of Science, Yerevan, Republic of Armenia

Tsaturyan Avetis,
Laboratory of BAS Purification and Certification at Scientific and Production Center "Armbiotechnology" NSPO,
National Academy of Science, Yerevan, Republic of Armenia

Minasyan Ela,
Laboratory of BAS Purification and Certification at Scientific and Production Center "Armbiotechnology" NSPO,
National Academy of Science, Yerevan, Republic of Armenia

Balabekyan Tsovinar,
Probiotics obtaining Sector, Scientific and Production Center "Armbiotechnology" NSPO,
National Academy of Science, Yerevan, Republic of Armenia

L-ARGININE SYNTHESIS BY LACTIC ACID BACTERIA

Abstract. The results of experimental and clinical studies indicate that L-arginine is a nutritionally essential amino acid for animal health. Additionally, L-arginine or L-citrulline may provide novel and effective therapies for obesity, diabetes, and the metabolic syndrome.

The screening of lactic acid bacteria (LAB) strains according to L-arginine synthesis showed significant intra-species differences. The 45 strains of LAB were investigated to L-arginine synthesis capability. It was shown, that some LAB strains (52%) are able to synthesized L-arginine (mg/ml). It was shown, that among 10 strains of *L. rhamnosus* species, 60% of strains are able to synthesized L-arginine. Two strains *Lactobacillus rhamnosus* 20–12 and *Lactobacillus plantarum* 66 shown increasing of L-arginine during growth in both nutrient media (MRS, milk). The *L. rhamnosus* 20–12

and *L.plantarum* 66 potential probiotic strains can be used as a basis of functional food product for application in veterinary and medicine.

Keywords: essential amino acids, probiotics, L-glutamic acid, functional food.

Introduction

Consumer interest in healthy lifestyle and health-promoting natural products is a major driving force for the increasing global demand of functional dairy foods. The health and therapeutic benefits of several probiotics is now well documented [1; 2]. Many types of bacteria have probiotic properties; however, the most documented groups comprise of lactic acid bacteria (LAB) and bifidobacteria, which are main microorganisms used for production of functional food [3]. So-called functional foods have been defined by the Food and Nutrition Board of the National Academy of Sciences as one that encompasses potentially positive effect on health, including "any modified food or food ingredient that may provide a health benefit beyond that of the traditional nutrients it contains [4].

The main function of the gut is to digest food and absorb nutrients. Has been an area of growing interest in gut microbiome research and appears to promote a health associated microbiota. Several observational studies have investigated the difference in the composition of the gut microbiota between those who are highly physically active (including athletes) and a range of other populations. Reported results include that a higher abundance of health-promoting bacterial species, increased microbiome diversity and greater relative increases in metabolic pathways (e.g. amino acid and antibiotic biosynthesis and carbohydrate metabolism) and fecal metabolites (e.g. microbial produced SCFAs; acetate, propionate, and butyrate) are associated with enhanced fitness [5].

According to Bergey's Manual of Determinative Bacteriology, majority of lactic acid bacteria are able to produce ammonia from L-arginine [6], but some of them are able to synthesize it [7]. It was shown, that L-arginine degraded by *Lactococcus lactis* subsp. *lactis* (the lactococcal subspecies known to catabolize arginine), pediococci, heterofermentative (*Lactobacillus brevis* and *L.fermentum*) and some unidentified homofermentative lactobacilli [7], for example, strains of *Lactococcus lactis* subsp. *lactis* from cheese, *Lact. leichmannii, Lact. plantarum* from fish Nonetheless. It is known the unique properties of the *L. helveticus* MP 12 strain to produce arginine amino acids in the milk [8]. Fermentation of milk by thermophilic lactic streptococci or acidophilic rods enriched the final products with at least 4 amino acids (cysteine, valine, proline, and arginine) [6]. Alanine and aspartate were utilized by some LAB and accumulated during the incubation of other LAB.

The results of experimental and clinical studies indicate that L-arginine is a nutritionally essential amino acid for human health [9]. Additionally, L-arginine or L-citrulline may provide novel and effective therapies for obesity, diabetes, and the metabolic syndrome. It has been shown that L-arginine stimulates the release of insulin and increases the sensitivity of β-cells to glucose, which is one of the main goals of the treatment of diabetes. At the same time, the biologically most important role of L-arginine is its use as a source of nuclear nitrogen for NO-synthase family enzymes since nuclear nitrogen plays an important role in many physiological and pathological processes in the body [10]. Only recently the hypothesis emerged that L-arginine is not only a substrate for the enzyme which converts it to NO and citrulline but also a nutrient whose intake is highly variable among individuals and among populations. The main question which is arising for nutritionists is the relevance of these new data for the long-term prevention of coronary heart disease, diabetes by manipulating dietary L-arginine and/or proteins from foods [11].

Until now there was no L-Arginine on the market registered as a feed additive, which is why Guanadi-

noacetic Acid (GAA) was used. This GAA is the precursor to creatine and is synthesized from arginine. Initially, GAA has been used to save the naturally occurring L-Arginine in some foods so that the animal can use it for other metabolic functions. However, there are studies that demonstrate the advantages of directly supplementing L-Arginine versus GAA [12].

During the creation of probiotic food and/or feed products, the content of essential amino acids in the final product is considering. The presence of probiotic properties of the LAB and the ability to synthesize L-arginine will improve the efficiency of the using of the selected strains during production of functional food products for application in veterinary and medicine.

The aim of the presented study was screening of lactic acid bacteria with probiotic properties according to L-arginine synthesis capability.

2. Materials and methods

2.1. Microbial strains and growth media Strains of lactic acid bacteria were isolated from samples of various dairy products made from milk of cow and other domestic animals, from rural households of several regions of Armenia. For cultivation of LAB strains the following nutrient media were used: **No 1.** MRS agar and broth (Merck (Germany), ISO (Italy), HiMedia (India)). **No 2**. Milk (1.5% of fat). **No 3.** The nutrient media prepared on the basis of curd whey with addition of the following salts ((%)± ± 0.2): $(NH4)_2SO4$–0.8; KH_2PO4–0.1; MgSO4––0.2; yeast extract-0,3; pepton-0,3; MnSO4–0.05; $CH_3COONa\times3H_2O$–0,2; pH=6.5±0.2 [13]. LAB strains maintained as frozen stock at –20 °C in the MRS broth containing 40% Glycerol. Before use, transferred twice into the appropriate medium and incubated during 24 hours in temperature controlled conditions in thermostat at 37 °C. *L.rhamnosus* 20–12 (MDC9631), strains of *Lactobacillus* genus (*L.casei, L.paracasei, L.acidophilus, L.plantarum, L.brevis*) and *Enterococcus* genus (*Ent. durans, Ent.faecium, Ent.faecalis*), used in presented work, were taken from the culture collection of Microbial Depository Center (MDC) of SPC "Armbiotechnology" NAS RA. LAB *L.rhamnosus* 8098, *L.rhamnosus* 8238, *L.rhamnosus* 6778, *L.rhamnosus* 6757, *L.rhamnosus* 4628 were taken from the culture collection of Russian National Collection of Industrial Microorganisms (VKPM) at Federal Institution "State Research Institute of Genetics and Selection of Industrial Microorganisms of the National Research Center" Kurchatov Institute", RF. Identification of LAB strains carried out by 16S rDNA gene sequencing method.

2.2. Obtaining of cell-free culture broth. Single colonies were grown in 5 ml of MRS broth (37 °C, 24 h) and when were transferred into 100 ml -Erlenmeyer's flask containing 50 ml of MRS broth, milk or media **No 3,** and incubated overnight at 37 °C in the thermostat. At the end of culture growth cell concentration achieved $(7.0 \pm 2) \times 10^8$ CFU/ml (of titration) and pH reduced to 3.5–4.2. Culture broth centrifuged at 6.000 g during 20 min and cell free culture broth (CFC broth) was obtained.

2.3. Determination of probiotic properties was carried out according to the generally accepted methods [3, 14]. Determination of ability to adhesion was carried out according to Henriksson et al. [15]. Antimicrobial activity of samples (aliquots of 40 μl) was assessed by spot-on-lawn method, measuring the size of the inhibition zone (diameter) of test culture growth (Ø, mm) after 24 h incubation in the thermostat at 30°C. The antimicrobial activity calculated according to Parente et al. [16] and expressed in arbitrary units (AU/ml).

2.4. Determination of L-arginine. L-arginine concentration was determined by the Sakaguchi reaction. To a 0.2 ml of sample, 2 ml 0.43 N NaOH, 0.5 ml dye, containing 8-hydroxyquinoline and 20 μl of dyacetil in propyl alcohol, were sequentially added. The solution was thoroughly mixed and absorbance read at 540 nm after 20 min of incubation. Values of O.D. 540 nm were compared with an L-arginine standard curve to estimate concentrations [17].

2.5. Analysis of Free amino acids profile in the CFC broth and final products was assayed according

to Ojinnaka and Ojimelukwe [18] on the Nexera X2 SHIMADZU (Shimadzu Corporation). Mobile phase: solution A (20 mmol potassium phosphate (pH=6.9) and solution B (45/40/15 ACN/ AOH/ H2O). Flow rate: 0.8, To C30 °C. Mixture of 17 amino acids (Sigma, St. Louis, MO, USA) was used as a control solution.

2.6. Statistical analysis. Microsoft Word 10 and Microsoft Office Excel 2010 applications have been used, the data received is valid for $p < 0.05$.

Results

The probiotic properties of the endemic strains of lactic acid bacteria, isolated from different dairy products from several regions of Republics of Armenia were shown [19–20]. It was shown, that LAB strains with probiotic properties were mainly presented with several species of *Lactobacillus* and *Enterococcus* genera [21–23].

To determine the presence of L-arginine synthesis, strains (about 45) of different species of the *Enterococcus* and *Lactobacillus* genus were investigated during the incubation on 3 different nutrient media. Results of study shown, that about 60% of LAB did not utilized L-arginine during the growth in the MRS synthetic nutrient media, and only 20% of strains produce L-arginine. During the growth in milk (1.5% fat) the increasing of L-arginine content observed for 52% of LAB strains, while growth in the synthetic nutrient media on the basis of milk whey (No 3) brings to increasing of L-arginine average up to 23%. Thus, it was shown, that synthesize of L-arginine depends on the nutrient media composition. The results of investigation of L-arginine synthesis by identified strains of LAB *Enterococcus* and *Lactobacillus* genus are summarized in (Table 1). As it seen from the given results, LAB strains showed intra-species differences according to L-arginine synthesis. Only two strains of *Lactobacillus* genus (*L. rhamnosus* BTK 20–12 and *L.plantarum* 66) and *Enterococcus* genus (*Ent. durans* M 42 and *Ent. faecium* M 44) shown increasing of L-arginine during the growth in both nutrient media (MRS, milk).

Table 1. – Comparative study of L-arginine synthesis by LAB strains

LAB of *Lactobacillus* genus	**L-arginine, mg/ml**		**LAB of *Enterococcus* genus**	**L-arginine, mg/ml**	
	Milk	**MRS**		**Milk**	**MRS**
Control (media)	0.7	0.4	*Ent. durans* K 13	1.0	0.9
L.rhamnosus 20–12	2.5	2.0	*Ent. durans* M 42	2.0	0.7
L. casei AG 31–2–2	0.35	0.85	*Ent. durans* M 22	1.8	0.5
L.plantarum 66	2.0	1.7	*Ent. durans* AG 76	0.35	0.75
L.acidophilus 1991	1.5	0.52	*Ent. durans* AA 11–6	0.35	0.5
L.brevis AG 31–9	0.45	0.65	*Ent. faecium* M 44	2.0	1.0
L. casei AG 35–2–1	0.95	1.2	*Ent. faecium* AG 53–11	0.55	0.5
L.paracasei AV 17–9	0.3	0.9	*Ent. faecium* 64	1.5	0.5
L. casei AV 38–1–1–1	0.4	1.0	*Ent. faecalis* AG 32–6	0.2	1.2
L.acidophilus Er-2317/402	1.2	0.9	*Ent. faecalis* AV 222	0.4	0.8

There are enough research papers showing the useful properties of different strains of *Lactobacillus rhamnosus* species. Presently, the only probiotic LAB strains clinically shown to have an effect are *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* when administered intra-vaginally once weekly or twice daily orally, reduced recurrences of UTI and restored a normal lactobacilli dominated vaginal flora in patients. *Lactobacillus rhamnosus* GG (LGG) can exert anti-inflammatory effects in the gut. *Lactobacillus rhamnosus* HN001 are probiotics that have been isolated from the human gastrointestinal tract [3].

Previously, several probiotic properties of *Lactobacillus rhamnosus* 20–12 were shown by us. It was shown, that antimicrobial activity against antibiotic resistant pathogens was due to by the presence of two low molecular weight (LMW) bacteriocins in the CFC culture liquid (BCN1–1470 Da and BCN2–670 Da) [13].

It is known that the population of microorganisms is heterogeneous and within one population (one strain) may be contained cells with varying physiological and biochemical properties. For example, it was shown, that LAB colonies of *Enterococcus faecium* strain may differ from each other by the content of the plasmids [24]. It has been suggested that the population variability on the expression of for the bacteriocin producer strain may also be heterogeneous. For increasing the yield of production of L-arginine by *L. rhamnosus* 20–12 the random method of gradual autoselection of single colonies according to L-arginine increasing was used (the standard method in genetics).) The same method was used for obtaining the strain shown over synthesis of L-arginine. The increasing of L-arginine yield occurs up to 40%.

The changes in free amino acid profile in the CFC culture liquid during the growth of *L. rhamnosus* 20–12 strain in two nutrient media were investigated. In the Fig. 1 the chromatograms of the profile of free amino acids after cultivation of *L. rhamnosus* 20–12 strain shown. As it seen from the given results, level of glutamic acid and L-arginine, calculated in mg /ml much higher than other amino acids. It is known that L-Glutamic acid (glutamate) is as a major excitatory neurotransmitter in the human brain and in the spinal cord. Glutamate is the principal mediator of sensory information, motor coordination, emotions and cognition, memory formation and retrieval. This amino acid has been used to help treat Parkinson's, fatigue, mental retardation, schizophrenia, muscular dystrophy, and alcoholism [5]. For increasing of the content the L-arginine and L-glutamic acid in the dairy products, in the technology of production of dairy products chemically purified amino acids usually added to the final product. Addition of L-arginine and/or L-glutamine improved health promoting characteristics and stable taste, increased vitality of lactic acid bacteria [25; 26].

The investigation of the content of two amino acids in 100 grams of fermented milk product after growing under the conditions of factory production of dairy products was carried out. The table shows (Table 2) the calculated data of those two mentioned above amino acids in the final products. The results of investigation are summarized in (Table 2). Obtained data was compared with the content of L-arginine in the therapeutic-dietary fermented sour milk product «Narine», developed on the basis of *Lactobacillus acidophilus* n.v. Ep 317/402 strain (certificate of patent of the USSR, No 163573, 1964) and produced in Republic of Armenia and Russian Federation. Therapeutic properties of acidophilic bacteria of "Narine" are confirmed with many years of clinical trials in Armenia, Russia, Ukraine, Latvia, Moldavia and Japan. Moreover, Japanese scientists ("Miki corporation" firm, Osaka, Japan) found that "Narine" bacteria contribute to the production of alpha, beta, and, especially, gamma-interferon increasing the immunity of the human organism thus serving a prophylactic means against many diseases. At present "Narine" product is widely used for dysbacteriosis treatment in newborns [27].

Table 2. Comparative evaluation of product (100 g of product)

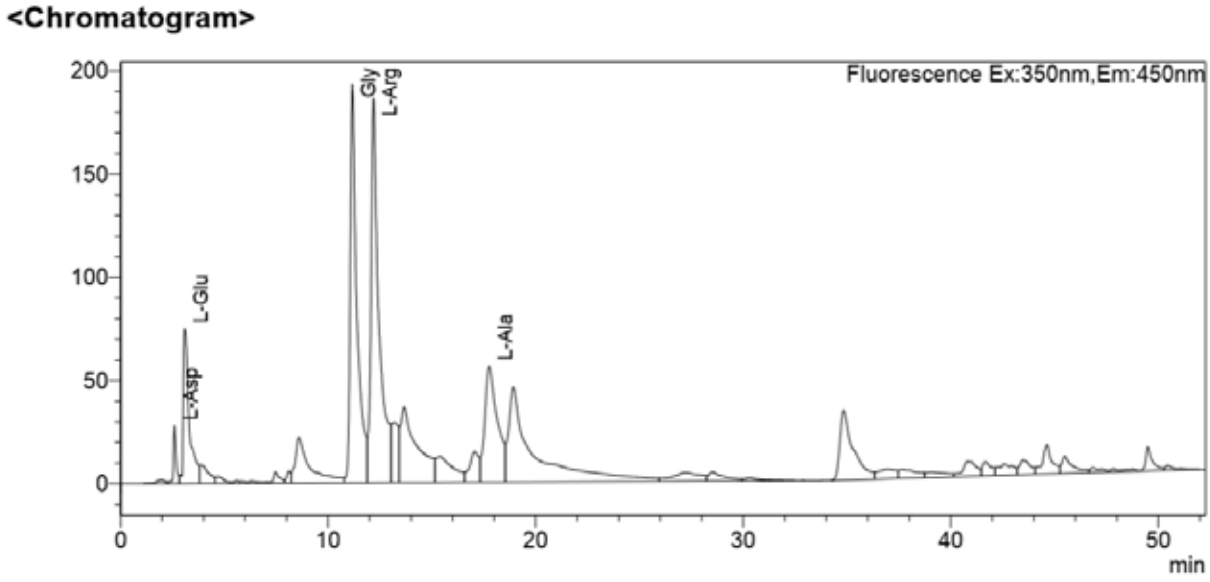
LAB strains	Amino acid content in product (mg per 100ml)		Acidity, °T	Cell count, CFU/ml	Antimicrobial activity AU/ml
	L-glu	L-arg			
L.acidophilus Er-2317 / 402	3.0	2.4	120 ± 10	1.8×10^9	1200
L.rhamnosus 20–12	3.0	6.6	100 ± 10	5.9×10^9	1000
Cow milk whole (control)	0.8	0.1	18 ± 5	none	none

As it seen from the given results, the fermented milk product «Narine» is inferior in these characteristics (glu and arg), which gives the prospect of using *L.rhamnosus* 20–12 strain.

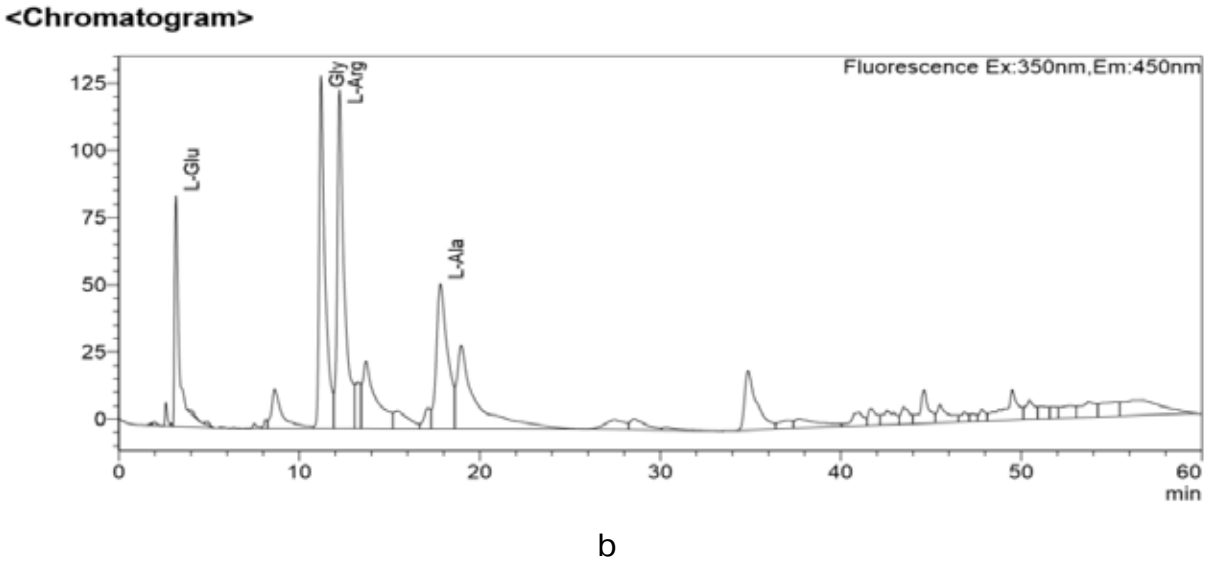
Thus, the role of L-arginine in the human diet and, in particular, diabetes is known and this makes the *L.rhamnosus* 20–12 strain promising for widespread use.

Discussion

Arginine is also considered to be a conditionally essential amino acid. Arginine has a wide range of biological functions and participates in tissue cell proteins formation within the body. Moreover, also take part in the synthesis of urea, creatine, creatinine, NO, glutamine and pyrimidine [10].



a



b

Figure 1. Free amino acid profile for L.rhamnosus 20–12 (a – milk, b – MRS)

L-Arginine is a basic amino acid in physiological fluids. For example, arginine deficiency in preterm infants, resulting in hyperammonemia and multiorgan dysfunction. Studies on fertility in both males and females shown, that oral administration of arg-HCl (0.5 g/day) to infertile men for 6–8 weeks markedly

increased sperm counts and motility in most patients and resulted in successful pregnancies. Dietary supplementation with 1 1.3% Arg-HCl to the diet for female rats either throughout the entire pregnancy (21 days) or between days 1 and 7 of gestation increased embryonic survival and birth litter size by 30% [10].

Today the production of probiotics or functional food products on the basis of new strains of LAB with selected properties or their combination is absent in the Republic of Armenia. At the same time, the isolation of new strains of endemic LAB and their investigations carried out by us during ast decade. More than 400 endemic strains of lactic acid bacteria, isolated from different dairy products from several regions of Republics of Armenia were investigated. It was shown, that some of them possessed probiotic properties [19; 23]. They have high antimicrobial and antioxidant activity, growth rate; differ by their basic probiotic properties. Some strains showed increased synthesis of arginine, which is important for treatment of diabetes. Comparing the content of L-arginine in some food products we can see that the obtained fermented dairy product is comparable by the content of L-arginine per 100 g of other L-arginine rich food products [10].

So, the use of the *L.rhamnosus* 20–12 strain as a basis of probiotic product in functional nutrition and its ability to adhere to the epithelial cells of GIT will make it possible to create a biofilm, which synthesizing L-arginine directly in the digestive tract.

The use of bacteria with certain selected properties (synthesis of essential amino acids, antimicrobial activity, probiotic properties) as starter culture becomes an effective alternative strategy to production of new functional feed additives with broader therapeutic and prophylactic properties, such as increased content of several amino acids (arginine, glutamic acid).

Thus, the role of L-arginine in the animal feeding is known and this makes the *L. rhamnosus* 20––12 strain promising for widespread use.

Ethical Approval. This article does not contain any studies with human participants or animals performed by any of the authors."

Conflict of Interest. The authors declare that they have no conflict of interest.

Data availability. All data generated or analyzed during this study are included in this published article and available from the corresponding author on reasonable request.

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