



Journal of Experimental Biology and Agricultural Sciences

<http://www.jebas.org>

ISSN No. 2320 – 8694

BREEDING PROBIOTIC STRAINS OF MICROORGANISMS

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Received – December 27, 2017; Revision – March 20, 2018; Accepted – April 04, 2018
Available Online – April 25, 2018

DOI: [http://dx.doi.org/10.18006/2018.6\(2\).335.341](http://dx.doi.org/10.18006/2018.6(2).335.341)

KEYWORDS

Breeding
Strain
Microorganisms
Probiotic
Properties
Identification.

ABSTRACT

This research was aimed at isolating pure cultures of microorganisms that fermented lactose from the contents of the digestive tract of wild animals, and at studying their technological properties. Further, the contents of the digestive tract of wild animals such as Caucasian wild goat, European roe deer and brown bear were procured from the hunters of the Republic of North Ossetia-Alania of the Russian Federation. Pure cultures of the lactose fermenting strains of microorganisms isolated from their digestive tracts, identified and deposited at the National Bioresource Center “The All-Russian Collection of Industrial Microorganisms” of the Federal State Budget Institution “GosNII Genetika” of the Ministry of Education and Sciences of the Russian Federation, Moscow. Various microbial strains such as *Enterococcus thailandicus* VKPMV-11826, *E. durans* VKPMV-11827, *E. mundtii* VKPMV-11828, *E. hiraе* VKPMV-11829, *E. faecalis* VKPMV-11830, *E. hiraе* VKPMV-11831, *E. thailandicus* VKPMV-11832, *E. thailandicus* VKPMV-11833, *E. hiraе* VKPMV-11834, *E. hiraе* VKPMV-11835 and *E. durans* VKPMV-1196 have been isolated from the digestive tract of various studied organisms. It has been found that the rate of milk coagulation by cultures of the studied strains of microorganisms bred in the Research Institute of Biotechnology of the Gorsky State Agrarian University, Republic of North Ossetia–Alania of the Russian Federation, is 6-8 hours, and the maximum limit of acid formation is 129 - 216°T on the 10th day. The studies have shown that lactic acid bacteria are very widespread both in the environment and in the body of animals, including wild animals.

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Peer review under responsibility of Journal of Experimental Biology and Agricultural Sciences.

Production and Hosting by Horizon Publisher India [HPI]
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1 Introduction

Currently, with the intensive development in biotechnology of food products including functional food, it is undeniably necessary to search for and develop scientific approaches to obtain probiotic products with the use of physiologically active microbial strains that ferment lactose and can suppress development of pathogenic or conditionally pathogenic microorganisms in the organism and improve digestibility of the components of the diet, and normalize the microflora of the digestive tract (Tsugkiev et al., 2016a). Identification of the microflora is of great importance for the research related to the food industry. In the microbiology of food products, exaction and identification is important for selecting a new starter strain, for labeling the product to which the starter is added, and for testing food products for the presence of undesirable organisms, e.g., pathogens (Hartman et al., 2001, Beasley, 2010; Zugkiev, 2016).

Further, according to Solovyeva (2010) searching of new microbial strains that form lactic acid is a promising and important field of biotechnology for creating functional food products. To increase the therapeutic and preventive action of functional food products, probiotic bacteria are widely used (Tsugkiev et al., 2015; Tsugkiev et al., 2016b).

Similarly, Tarakanov (2000) and Tarakanov (2002) also suggested the vital role of probiotic bacteria in medicinal biotechnology and suggested that these can be used for the gradual replacement of traditional preparations in certain medical cases. Probiotic lactic acid microorganisms can be efficiently used in breeding young stock of agricultural animals and poultry (Tarakanov, 1999).

Production of probiotic products is essential for preventing and treatment of malfunctioning digestive tract (Sizenko, 2005). Bartosch et al. (2004) have reported that the microbial population in the intestines undergoes certain changes in the process of human development. Therefore, restoration of the normal biocenosis of the intestines using probiotics is important. According to Buryaco (2004) use of probiotics is the most efficient and physiological method of preventing and treating dysbacteriosis of the digestive system. Obtaining such products is extremely relevant for the ecologically unfavorable regions of Russia (Kharitonov, 2003). Further, Gill et al. (2006) reported effect of probiotics on the composition of the intestinal flora in elderly people to be a means of improving the intestinal immune system and the immune system of the organism as a whole. Probiotics composed of symbiotic strains of bacteria, aerobes and anaerobes have multidimensional and powerful immunomodulatory effect than the single-component preparations (Fitzpatrick et al., 2007).

Enterococci are typical representatives of the normal microflora of the gastrointestinal tract of vertebrates, they are among the first to colonize the newborn. Their role is especially important for ensuring colonizational resistance of mucous membranes (Beasley 2010). The main condition for classifying strains of enterococci as probiotics is the fact that they are to be bred for creating food products (Fisher & Phillips, 2009). Trishina et. al. (2017) defined the generic and species belonging of the family Enterobacteriaceae isolated from the surface water bodies in city Rostov-on-Don according to the results of several biochemical studies using the MALDI Biotype application.

Obtaining high quality bacterial preparations required constant work on growing and breeding new strains of microorganisms with required properties for each kind of starter preparation (Tsugkiev et al., 2012). Therefore, present study has been carried out for the searching of new biologically active probiotic strains of microorganisms and development of efficient schemes of their implementation.

2 Materials and Methods

This research was aimed to isolate pure cultures of lactose fermenting microorganisms from the contents of the digestive tract of wild animals and studying their technological properties. Digestive tracts of Caucasian wild goat, European roe and brown bear were investigate for the colonization of member of Enterococci. Pure cultures of the isolated microorganisms was identified and deposited at the National Bioresource Center “The All-Russian Collection of Industrial Microorganisms” of the Federal State Budget Institution “GosNIIGenetika” of the Ministry of Education and Science of Russia, Moscow.

For primary isolation of lactose fermenting microorganisms from samples of the digestive tract of wild animals living in the forests and mountains of the Republic of North Ossetia-Alania, anaerobic state for cultivating microorganisms was created in micro anaerobic jars. Low-fat cow milk was used as isolating medium and the pH of this isolating medium was adjust to 5. Milk sample used for isolation were sterilized for 30 minute at 0.5 atm to suppress, this process will stop the growth of other microorganisms that did can develop at this pH.

Bacterial strains that could ferment lactose was isolated by introducing a bacteriological loop having content of the digestive tract of wild animals into tubes with sterile defatted milk of the samples and incubated at 28°C, 37°C and 45°C for 24 hours. Transplantation of microorganisms into sterile milk was made daily until obtaining a clot of milk without clot breaks, gas bubbles and buttermilk sedimentation.

Isolated microorganal colonies were cultured on Lactobacillus MRS Agar (HiMedia Laboratories Pvt. Ltd., India) for obtaining their pure cultures. Initially isolated microorganism were identified on the basis of cultural, morphological, tinctorial, physio-biochemical and technological characteristics. Identification was carried out with the help of manual developed by Bannikova (1975) and Handbook of microbiological and virological research methods (1982). Further, this identification was based on the size, texture, shape and the color of the colonies; morphology of cells of the microorganisms; Gram staining; growth at different pH and temperatures; gelatin liquefaction; formation of ammonia from arginine; ability to produce acetylmethylcarbinol (the Voges-Proskauer reaction); disposal of carbohydrates and alcohols: glucose, galactose, lactose, sucrose, maltose, mannitol, arabinose, xylose, raffinose, sorbitol, glycerin, dextrin, starch and antagonistic properties towards representatives of harmful microflora.

Technological properties of pure cultures of the studied strains of microorganisms were determined by considering the milk fermentation rate (active acidity), maximum acid-producing ability (°T), and the CFU number in 1 ml of fermented milk using the serial dilution method.

Generic belonging of pure cultures of microorganisms was confirmed by analysis of the sequences of variable sections of genes encoding 16S rRNA at the National Bioresource Centre "The All-Russian Collection of Industrial Microorganisms" of the Federal State Budget Institution "GosNIIGenetika" of the Ministry of Education and Science of Russia, Moscow.

3 Results and discussion

Results of bacterial isolation along with their concern indices of strains and sources of obtaining have been represented in Table 1. From the content of caucasian wild goat intestines five *Enterococcus* species viz., *E.durans* VKPM V-11827, *E.hirae* VKPM V-11829, *E.thailandicus* VKPM V-11832, *E. thailandicus* VKPM V-11826 and *E.mundtii* VKPM V-11828 have been isolated and identified. Further, from the intestines content of European roe only two *Enterococcus* species (*E.durans* VKPM V-11960 and *E.hirae* VKPM V-11835) have been isolated while in case of brown bear intestines' contents four different strains of *Enterococcus* viz.,- *E.hirae* VKPM V-11834, *E.hirae* VKPM V-11831, *E. thailandicus* VKPM V-11833 and *E.faecalis* VKPM V-11830 have been isolated and identified.

In the identified strains of microorganisms, a set of cultural, morphological, tinctorial, physiological, biochemical and technological properties was established and these results are in agreement with the findings of Trishina et al. (2017). The morphological characteristics of the studied microorganism are important in the determining its species belonging. Result of morphological, ability of spores formation, tinctorial properties and agility have been presented in table 2.

All studied strains of microorganisms are facultative anaerobes (anaerobic condition was created in microanaerobic jars), have cocci-like shape of cells and are not agile, gram-positive, without spore formation, arranged into diplococci and short chains. Study of the cultural properties of the strains revealed that studied

Table 1 Strains of microorganisms obtained from various wild animals intestine

No.	Bacterial Strain	Collection number of the strain	Animal whose intestines were used for the isolation of bacterial strain
1.	<i>E. durans</i>	VKPM V-11827	Caucasian wild goat
		VKPM V-11960	European roe
2.	<i>E. hirae</i>	VKPM V-11834	Brown bear
		VKPM V-11835	European roe
		VKPM V-11829	Caucasian wild goat
		VKPM V-11831	Brown bear
3.	<i>E. thailandicus</i>	VKPM V-11826	Caucasian wild goat
		VKPM V-11832	Caucasian wild goat
		VKPM V-11833	Brown bear
4.	<i>E. mundtii</i>	VKPM V-11828	Caucasian wild goat
5.	<i>E. faecalis</i>	VKPM V-11830	Brown bear

Table 2 Morphology of the studied strains of microorganisms

Strain	Shape, arrangement, spores	Agility
<i>E. durans</i> VKPM V-11827	Diplococci, without spores	-
<i>E. durans</i> VKPM V-11960	Diplococci, without spores	-
<i>E. hirae</i> VKPM V-11834	Cocci arranged a short chain, without spores	-
<i>E. hirae</i> VKPM V-11835	Cocci arranged a short chain, without spores	-
<i>E. hirae</i> VKPM V-11829	Short chains of cocci and diplococci, without spores	-
<i>E. hirae</i> VKPM V-11831	Short chains of cocci and diplococci, without spores	-
<i>E. thailandicus</i> VKPM V-11826	Diplococci and short chains of cocci, without spores	-
<i>E. thailandicus</i> VKPM V-11832	Diplococci and chains of cocci, without spores	-
<i>E. thailandicus</i> VKPM V-11833	Diplococci and chains of cocci, without spores	-
<i>E. mundtii</i> VKPM V-11828	Diplococci and chains of cocci, without spores	-
<i>E. faecalis</i> VKPM V-11830	Diplococci, without spores	-

cultures has small sized colonies with convex profile, rounded shape, soft texture, fine structure and white color.

Data given in Table 3 revealed that all studied microorganisms strains cultures were capable to growing at 40 and 45°C temperatures in hydrolyzed milk containing 20% of bile and 6.5% NaCl. Further, it was reported that all studied strains could survive at the temperature of 60°C for 30 min. Further, pH value also affect the multiplication of isolated microorganism and results of study revealed that bacterial strains of *E. hirae* VKPM V-11834, *E. thailandicus* VKPM V-11826, *E. thailandicus* VKPM V-11832 and *E. thailandicus* VKPM V-11833 can grow at the pH 9.6.

It has been established that cultures of all strains form ammonia from arginine, ferment glucose, galactose, lactose, maltose and dextrin. It was reported that isolated strains *E. hirae* VKPM V-11835, *E. hirae* VKPM V-11829, *E. hirae* VKPM V-11831 and *E. mundtii* VKPM V-11828 can also fermented raffinose. Further, mannitol and sorbitol fermentation properties were reported from the bacterial strain of *E. mundtii* VKPM V-11828 and *E. faecalis* VKPM V-11830. In addition, bacterial strain *E. mundtii* VKPM V-11828 can fermented arabinose and xylose and is able of forming a yellow pigment.

The most important property of lactic acid fermenting microorganisms is their ability to form acid, which is the basis of the time of storage of a probiotic product. The technologically important indicator of a lactic acid microorganism is the milk coagulation rate. Production-valuable properties of the obtained strains of microorganisms are shown in Table 4.

The rate of milk coagulation by studied microorganisms bred at Research Institute of Biotechnology of the Gorsky State Agrarian University suggested that isolated microorganisms bred took 6-8 hours, while the maximum limit of acid formation is 129 - 216°T on the 10th day.

The content of CFU bacteria in probiotic products is the most important consumer indicator. Studied microorganisms have very high microbial count in fermented milk sample and it is equal to 10⁹ - 10¹⁰CFU/ml and more, which is the basis for the antagonistic activity of the studied microorganisms in terms of harmful microorganisms. Further, probiotic properties are an important indicator that could be use as a strong basis for assessing antagonistic activities of this isolated microbial strain (Table 5).

Isolated strains had varying antagonistic activity against the pathogenic and conditionally pathogenic microflora, which should be paid attention during the preparation of bacterial starters for probiotic products and dairy products of functional purpose for preventive care and treatment of digestive tract dysfunction.

The zone of growth inhibition of *Escherichia coli* ranged between 25 and 31 mm, and it was reported between 23 and 30 mm for *Staphylococcus aureus*. The highest antagonistic activity was exhibited by strains of *E. durans* VKPM V-11960, *E. hirae* VKPM V-11834 and *E. thailandicus* VKPM V-11832.

The results of identifying strains of microorganisms obtained from the contents of the digestive tract of wild animals living in the Republic of North Ossetia-Alania have been confirmed by analyzing sequences of variable sections of genes encoding 16S

Table 3 Results of identification of the studied strains of microorganisms

Tests		Species and strains of microorganisms										
		<i>E. durans</i> VKPM V-11827	<i>E. durans</i> VKPM V-11960	<i>E. hirae</i> VKPM V-11834	<i>E. hirae</i> VKPM V-11835	<i>E. hirae</i> VKPM V-11829	<i>E. hirae</i> VKPM V-11831	<i>E. thailandicus</i> VKPM V-11826	<i>E. thailandicus</i> VKPM V-11832	<i>E. thailandicus</i> VKPM V-11833	<i>E. mundtii</i> VKPM V-11828	<i>E. faecalis</i> VKPM V-11830
Gram staining:		+	+	+	+	+	+	+	+	+	+	+
Growth in milk (Temperature)	40°C	+	+	+	+	+	+	+	+	+	+	+
	45°C	+	+	+	+	+	+	+	+	+	+	+
Growth in milk (pH)	9.2	-	-	+	±	±	±	+	+	+	-	-
	9.6	-	-	+	±	±	±	+	+	+	-	-
Growth in hydrolyzed milk containing NaCl (%)	2	+	+	+	+	+	+	+	+	+	+	+
	4	+	+	+	+	+	+	+	+	+	+	+
	6.5	+	+	+	+	+	+	+	+	+	+	+
Growth in hydrolyzed milk containing 20% of bile:		+	+	+	+	+	+	+	+	+	+	+
Growth in milk containing methylene blue (%)	0.01	+	+	+	+	+	+	+	+	+	+	+
	0.1	+	+	+	+	+	+	+	+	+	+	+
NH ₃ formation from arginine		+	+	+	+	+	+	+	+	+	+	+
Gelatin liquefaction		-	-	-	-	-	-	-	-	-	-	-
The Voges-Proskauer reaction				+	+	+	+				+	
Heating for 30 minutes (temperature °C)	55	+	+	+	+	+	+	+	+	+	+	+
	60	+	+	+	+	+	+	+	+	+	+	+
Fermentation of carbohydrates and alcohols	Glucose	+	+	+	+	+	+	+	+	+	+	+
	Galactose	+	+	+	+	+	+	+	+	+	+	+
	Lactose	+	+	+	+	+	+	+	+	+	+	+
	Sucrose	-	+	-	+	+	+	+	+	+	+	+
	Maltose	+	+	+	+	+	+	+	+	+	+	+
	Mannitol	-	-	-	-	-	-	-	-	-	+	+
	Arabinose	-	-	-	-	-	-	-	-	-	+	-
	Xylose	-	-	-	-	-	-	-	-	-	+	-
	Raffinose	-	-	-	+	+	+	-	-	-	+	-
	Sorbitol	-	-	-	-	-	-	-	-	-	+	+
	Glycerin	+	-	-	-	+	+	-	-	-	+	+
	Dextrin	+	+	+	+	+	+	+	+	+	+	+
Starch	-	-	-	-	-	-	-	-	-	-	-	

rRNA at the National Bioresource Centre “The All-Russian Collection of Industrial Microorganisms” of the Federal State Budget Institution “GosNIIGenetika” of the Ministry of Education and Science of Russia, Moscow with allocation of collection numbers. All studied strains are protected by Russian Federation patents for invention.

It has been found that the rate of milk coagulation by cultures of the studied microorganisms bred is 6-8 hours, while the maximum

limit of acid formation is 129 - 216°T on the 10th day. Further, the results of study suggested that lactose fermenting enterococci are very widespread in the environment and animals both.

The relevance of our research in isolating and identifying strains of enterococci with probiotic properties has been confirmed by the opinion of Beasley (2010), who also believes that enterococci are typical representatives of normal microflora of the gastrointestinal tract of man and many vertebrates and have important role in

Table 4 Technological properties of studied bacteria

Strain	Milk coagulation rate (h)	Acidity of fermented milk, °T:	
		Active at the moment of milk coagulation	Maximum on the 10 th day
<i>E. durans</i> VKPM V-11827	7	73	201
<i>E. durans</i> VKPM V-11960	6	71	198
<i>E. hirae</i> VKPM V-11834	8	72	213
<i>E. hirae</i> VKPM V-11835	8	69	216
<i>E. hirae</i> VKPM V-11829	6	75	129
<i>E. hirae</i> VKPM V-11831	8	72	202
<i>E. thailandicus</i> VKPM V-11826	6	67	178
<i>E. thailandicus</i> VKPM V-11832	7	70	210
<i>E. thailandicus</i> VKPM V-11833	8	69	130
<i>E. mundtii</i> VKPM V-11828	7	68	136
<i>E. faecalis</i> VKPM V-11830	8	81	138

Table 5 Antagonistic activity of strains of microorganisms (by the method of diffusion in agar)

Tested strain	Test microbe	
	<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>
	The zone of the test microbe growth inhibition (mm)	
<i>E. durans</i> VKPM V-11827	27	23
<i>E. durans</i> VKPM V-11960	30	27
<i>E. hirae</i> VKPM V-11834	31	27
<i>E. hirae</i> VKPM V-11835	29	25
<i>E. hirae</i> VKPM V-11829	28	26
<i>E. hirae</i> VKPM V-11831	29	25
<i>E. thailandicus</i> VKPM V-11826	25	28
<i>E. thailandicus</i> VKPM V-11832	28	30
<i>E. thailandicus</i> VKPM V-11833	26	29
<i>E. mundtii</i> VKPM V-11828	28	25
<i>E. faecalis</i> VKPM V-11830	25	27

ensuring colonizational resistance of the mucous membranes. The need for the studies of breeding in new strains of enterococci has been confirmed by the results of Fisher & Phillips (2009) believed that the main condition for classifying strains of enterococci as probiotics is the fact that they are to be bred for creating food products. Isolated microorganism have capacity to produced various probiotics which can be used as strater food for various other microorganisms. These results are confirmed findings of Sizenko (2005) who believed that production of probiotic food is essential for preventing and treating digestive tract dysfunctions. Since the use of probiotics is the most efficient and physiological method of preventing and treating dysbacteriosis of the digestive system (Buryako, 2004).

Conclusion

Results of present study have shown that lactic acid bacteria are very widespread both in the environment and in the organisms of animals, including wild animals. These microorganisms have wide range of characteristics and these are varies with the organisms. Further, bacterial strains identified in present study can be use for the production of functional probiotics and dairy products.

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