Andrey Georgievich Koshchaev et al /J. Pharm. Sci. & Res. Vol. 9(8), 2017, 1373-1379

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Screening of Microorganism Symbiont Strains as a Base of Probiotics for Poultry Industry

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Abstract

This work discusses screening of microorganism symbiont strains in terms of their morphological, cultural, and biochemical properties in order to include them into developed three-strain probiotic food additive for poultry operation. Mobility, Gram stain, shape and location of cells, existence and location of spores, behavior towards oxygen, optimum growth temperature were analyzed, sugar fermentation spectra, resistance to bile and phenol solution, vitamin requirements, and acid formation of the considered cultures were determined. Antagonistic activity of the considered bacteria strains was determined on solid nutrient media by deferred antagonism method. The antibiotic susceptibility of strains was determined with regard to seven medications by diffusion to agar using standard disks. Interrelation between constituent strains of feed additive was studied by their simultaneous inoculation on solid and liquid nutrient media, incubation and CFU count by limiting dilution method. Submerged cultivation of the bacterial additive components was developed on the basis of nutrient medium with derived products of raw animal and plant materials. The experimental results demonstrated that among the used collection strains of lactic acid bacteria the following collection strains were characterized by high acid forming and antagonistic ability, resistance to phenol, bile and some antibiotics, as well as ability to ferment various carbohydrates: *Lactobacillus acidophilus* B-8634, *Lb. delbrueckii* subsp. *bulgaricus* B-6543, *Streptococcus thermophiles* B-2894. On the basis of selected microorganism cultures as well as derived products of raw animal and plant materials a liquid probiotic food additive was engineered and developed, possessing high antagonistic properties, and its microflora was able to survive in gastroenteric tract.

Keywords: Lactobacteria, morphology, stability, antagonism, nutritive medium, titer, probiotic additive.

INTRODUCTION

The important task of food supply to population is directly related with development of cattle breeding and poultry operation in particular. Urgent purposes of Russian governmental agrarian policy in the period up to 2020 are provision of demands for agrarian production and efficient import substitution at cattle market. In 2020 it is planned to reduce the import portion of meat products to 13%, milk import – to 12%. Meat diet will be completely satisfied by Russian products. This requires for implementation of innovative technologies for maintenance and feeding of farm livestock [9, 15, 20].

Under conditions of intensive commercial poultry operation, when limited sites are used for breeding of large poultry stock, there is high risk of occurrence of commensal and pathogenic microorganisms. Prolonged uncontrolled application of feed antibiotics in commercial poultry operation resulted in wide spread of gastrointestinal problems, which ranked next after virus diseases and were the main cause of death of growing stock [1, 3, 6, 8, 16].

Normal gut microflora participates in maintenance of colonization resistance of lining of intestines and plays an important role in prevention of human and animal diseases [7, 18, 24, 25]. Development of numerous pathologies of animals can be prevented by feeds enriched with biologically active food probiotic additives – live microorganism cultures. Their application exerts anti-infective, immunomodulatory impact on organism, improves barrier functions, stimulates motility and excretory functions of intestinal tract. Scientific background of engineering of probiotic medications is based on analysis of interactions between macro- and microorganisms. At present the concept of intestinal microbiota as of independent organ, which covers intestinal wall in the form of biofilm, is actively considered [2, 4, 11, 23]. The most widespread probiotics are lactic acid bacteria and bifidobacteria [10, 21, 22]. The aspects of application of probiotics cover wide range of issues related with correction of intestinal biocenosis, immune, hormone, and ferment system of growing and mature stock. Nowadays the most popular on the probiotic market are medications consisting of several bacterial strains (multicomponent) or including additives enhancing their action [12, 17, 19, 26].

Modern approach to development of probiotics and additives implies application of various microorganisms in certain combinations and production in the form allowing for their prolonged storage at ambient temperature. The medications should preserve their properties during preparation of formulated feed and additives. In order to improve digestibility and accessibility of feeds, stimulate growth and maturing of livestock, increase non-specific immunity the following medications are used: ferment, probiotic, prebiotic, and combined ferment-probiotic ones, as well as complex probiotics enriched with phytocomponents [5, 13, 14].

It follows from the aforementioned that development and implementation of biotechnological medications and additives, alternative to feed antibiotics, is

an urgent issue for solution of important problem of supply of environmentally safe livestock products.

This work is aimed at screening of microorganism symbiont strains, promising for use in probiotics, study of their morphological, cultural and biochemical properties, as well as engineering and development of laboratory production of three-strain probiotic food additive for sanitation and protection of farm poultry against harmful infectious diseases.

METHODS

Eleven collection strains of thermophilic microorganisms of *Streptococcus* and *Lactobacillus* species obtained from All-Russian Collection of Microorganisms (Moscow) were studied.

While developing three-strain probiotic food additive, the main cultural and morphological properties of the strains were studied (mobility, Gram stain, shape and location of cells, existence and location of spores, behavior towards oxygen, and optimum growth temperature).

While studying biochemical properties of lactic acid bacteria sugar fermentation spectra, resistance to bile and phenol solution were determined by addition of these compounds to liquid nutrient medium. Bacteria requirements of thiamine, riboflavin and folic acid were determined by the absence of growth in nutrient medium without food factor, and active acid formation of the considered strains was determined by titrimetric method and expressed in Turner degrees (°T).

Antagonistic activity of the considered bacterial strains was determined on solid nutrient media by deferred antagonism method with modifications by Lenzner. Antibiotic susceptibility of the strains was determined with regard to seven medications by diffusion to agar using standard disks.

Interrelation between constituent strains of feed additive was studied by their simultaneous inoculation on solid and liquid nutrient media, incubation and CFU count by limiting dilution method.

Titer of microorganisms in samples was determined by consecutive ten-fold dilutions of considered material in sodium chloride with their subsequent inoculation onto solid agar mediums, incubations at optimum growth temperature and counting of developed colonies on Petri dishes. The dishes with 150–250 colonies were counted.

Efficiency of selection of optimum composition of nutrient medium was accounted by number of microorganism cells. Submerged cultivation of the bacterial additive components was developed on the basis of nutrient medium with derived products of raw animal and plant materials.

RESULTS AND DISCUSSION

While engineering three-strain probiotic food additive eleven collection strains of thermophilic lactic acid bacteria of *Streptococcus* (*thermophiles* – 4 strains) and *Lactobacillus* (*bulgaricus* – 3 strains, *acidophilus* – 4 strains) were studied. At initial stage cultural and morphological properties of the considered strains were estimated; the results are summarized in Table 1.

As can be seen in Table 1, all considered strains exhibit peculiar features of lactic acid bacteria: they are gram positive and immobile, they are facultative anaerobes and in terms of morphology they are referred to this physiological bacterial group. The optimum growth temperature for all considered strains is 40°C.

An important physiological property of lactic acid bacteria is their ability to ferment carbohydrates (Table 2).

As can be seen in Table 2, the considered strains in this or that extent are able to ferment carbohydrates. Six of seven ferments *Lactobacillus acidophilus* B-8634 involved carbohydrates (cellobiose, fructose, lactose, raffinose, saccharose, xylose). As for *Lactobacillus bulgaricus* only one strain recovered four carbohydrates – B-6543. *Streptococcus thermophiles* B-2894 was able to recover six substrates during its growth (arabinose, cellobiose, fructose, lactose, saccharose, xylose).

Table 1. Cultural and morphological properties of lactic acid bacteria considered in this work

Strain	Gram stain/mobility *	Shape and location of cells	Behavior towards oxygen	Temperature optimum,°C
Lactobacillus acidophilus				
B-2104	+/–	Rods with rounded edges, located alone, chains	Facultative anaerobe	40
B-2366				
B-2846				
B-8634				
Lactobacillus delbrueckii subsp. bulgaricus				
B-2455	+/–	Rods with rounded edges, located alone, chains	Facultative anaerobe	40–43
B-6543				
B-7638				
Streptococcus thermophiles				
B-2894	+/–	Round, oval, and spherical cocci, located alone, in pairs or as chains of various length	Facultative anaerobe	40–42
B-3142				
B-3808				
B-5626				

Remark: * "+" – existing characteristic; "–" – non-existing characteristic.

Table 2. Carbohydrate fermentation by lactic acid bacteria

Strain	Fermentation *						
	arabinose	cellobiose	fructose	lactose	raffinose	saccharose	xylose
Lactobacillus acidophilus							
B-2104	–	–	+	+	–	+	–
B-2366	–	–	–	+	–	+	–
B-2846	–	+	+	+	–	–	–
B-8634	–	+	+	+	+	+	+
Lactobacillus delbrueckii subsp. bulgaricus							
B-2455	–	+	+	–	–	+	–
B-6543	–	+	+	–	+	+	–
B-7638	–	–	+	–	–	+	–
Streptococcus thermophiles							
B-2894	+	+	+	+	–	+	+
B-3142	–	+	–	+	–	+	–
B-3808	+	+	–	–	–	+	–
B-5626	–	+	+	+	–	+	+

Remark: * "+" – fermented; "–" –not fermented.

Table 3. Vitamin requirement of lactic acid bacteria

Strain	Requirement of *		
	thiamine	riboflavin	folic acid
Lactobacillus acidophilus			
B-2104	–	+	–
B-2366	+	+	+
B-2846	+	+	+
B-8634	–	+	–
Lactobacillus delbrueckii subsp. bulgaricus			
B-2455	–	+	–
B-6543	–	+	–
B-7638	–	+	–
Streptococcus thermophiles			
B-2894	–	+	–
B-3142	–	+	–
B-3808	–	+	+
B-5626	–	+	–

Remark: * "+" – required for growth; "–" – not required for growth.

Table 4. Resistance of lactic acid bacteria to bile and phenol

Strain	Growth in nutrient medium with bile,%			Growth in nutrient medium with phenol, 0.4%
	20	30	40	
Lactobacillus acidophilus				
B-2104	+	±	–	+
B-2366	+	+	±	±
B-2846	+	±	–	±
B-8634	+	+	+	+
Lactobacillus delbrueckii subsp. bulgaricus				
B-2455	+	+	+	+
B-6543	+	+	+	+
B-7638	+	±	–	±
Streptococcus thermophiles				
B-2894	+	+	+	+
B-3142	+	±	±	–
B-3808	+	±	–	±
B-5626	+	+	±	±

Remark: * "+" – good growth, "±" – weak growth, "–" – no growth.

While selecting probiont strain it is important to be aware of trophic abilities of microorganism with regard to such nutrient components as vitamins, since the lower is the rate of their consumption by strain, the less expensive is the formation of nutrient medium for accumulation of cell biomass. Vitamin requirement of the considered strains is summarized in Table 3.

The results in Table 3 demonstrate that the strain B-8634 of *Lactobacillus acidophilus* consumes only riboflavin for its growth, which is also used by all strain cultures of *Lb. delbrueckii* subsp. *bulgaricus*. Concerning the species of *Streptococcus thermophiles*, riboflavin is used only by three strains: B-2894, B-3142, and B-5626.

Ability of lactic acid bacteria to survive in gastroenteric tract and resistance to biological liquids of organism are important for probiotic properties of the bacteria. Resistance of microorganisms was evaluated visually by occurrence or absence of growth in liquid nutrient medium containing bile and phenol (Table 4).

Table 5. Antibiotic susceptibility of lactic acid microorganisms

Strain	Antibiotic						
	nystatin	erythromycin	levomycetin	neomycin	tetracycline	streptomycin	ampicillin
	Dosage, μg						
	15	15	30	30	30	30	10
	Inhibition zones of lactic acid cultures, mm						
Lactobacillus acidophilus							
B-2104	17 ± 0.5	10 ± 0.3	11 ± 0.4	10 ± 0.4	10 ± 0.4	8 ± 0.4	15 ± 0.6
B-2366	12 ± 0.4	17 ± 0.4	11 ± 0.5	15 ± 0.6	15 ± 0.7	14 ± 0.7	17 ± 0.5
B-2846	9 ± 0.4	8 ± 0.3	8 ± 0.3	11 ± 0.7	10 ± 0.5	8 ± 0.3	6 ± 0.2
B-8634	7 ± 0.4	7 ± 0.5	8 ± 0.4	10 ± 0.6	8 ± 0.7	5 ± 0.2	8 ± 0.4
Lactobacillus delbrueckii subsp. bulgaricus							
B-2455	11 ± 0.6	8 ± 0.6	11 ± 0.5	12 ± 0.5	8 ± 0.4	6 ± 0.3	9 ± 0.5
B-6543	9 ± 0.4	7 ± 0.6	10 ± 0.4	7 ± 0.5	9 ± 0.3	4 ± 0.1	5 ± 0.5
B-7638	15 ± 0.7	17 ± 0.6	11 ± 0.4	12 ± 0.5	15 ± 0.9	14 ± 0.6	17 ± 0.4
Streptococcus thermophiles							
B-2894	9 ± 0.7	7 ± 0.2	9 ± 0.4	8 ± 0.3	5 ± 0.5	6 ± 0.3	8 ± 0.1
B-3142	10 ± 0.4	8 ± 0.5	10 ± 0.3	11 ± 0.5	9 ± 0.6	7 ± 0.3	11 ± 0.8
B-3808	11 ± 0.5	10 ± 0.3	12 ± 0.3	14 ± 0.4	12 ± 0.5	11 ± 0.4	14 ± 0.3
B-5626	12 ± 0.6	10 ± 0.6	9 ± 0.3	8 ± 0.5	8 ± 0.7	8 ± 0.3	9 ± 0.5

It can be seen in Table 4 that maximum resistance to the used nutrient media was that of *Lb. acidophilus* B-8634, *Lb. delbrueckii* subsp. *bulgaricus* B-2455, *Lb. delbrueckii* subsp. *bulgaricus* B-6543 and *St. thermophiles* B-2894.

We studied susceptibility of the collection strains to therapeutic dosage of seven antibiotics (Table 5).

Evaluation of antibiotic susceptibility of the collection strain cultures demonstrated that the highest resistance to antibiotics among species of *Lactobacillus* was shown by two strains: *Lb. acidophilus* B-8634 and *Lb. delbrueckii* subsp. *bulgaricus* B-6543, and among *Streptococcus – by St. thermophiles* B-2894.

Acid formation of lactic acid bacteria is considered as integrated parameter characterizing biochemical and antagonistic activity. Normalized performance of specific activity of lactic probiotics is the activity of acid forming, hence, is one of important criterion of strain selection upon engineering of probiotics (Table 6).

Table 6. Titratable acidity of lactic acid bacteria,°T

Strain	Titratable acidity on milk	
	after 12 h	after 24 h
Lactobacillus acidophilus		
B-2104	89 ± 6	161 ± 10
B-2366	84 ± 9	157 ± 10
B-2846	98 ± 7	176 ± 12
B-8634	98 ± 6	187 ± 11
Lactobacillus delbrueckii* subsp. *bulgaricus		
B-2455	72 ± 6	179 ± 9
B-6543	96 ± 9	203 ± 11
B-7638	78 ± 7	189 ± 10
Streptococcus thermophiles		
B-2894	94 ± 5	201 ± 11
B-3142	86 ± 8	184 ± 10
B-3808	81 ± 5	188 ± 9
B-5626	88 ± 9	195 ± 14

Table 7. Antagonistic activity of lactic acid bacteria towards test cultures of pathogenic strains

Strain	Inhibition zones of test cultures, mm				
	S. flexneri	*S. sonnei*	*E. coli*	*S. aureus*	*S. enteridis*
Lactobacillus acidophilus					
B-2104	13.4 ± 1.0	9.7 ± 0.7	16.7 ± 0.9	10.5 ± 0.5	13.7 ± 0.6
B-2366	13.1 ± 0.7	11.6 ± 0.6	14.3 ± 1.7	11.6 ± 0.9	12.3 ± 0.6
B-2846	11.7 ± 0.4	10.7 ± 0.5	15.6 ± 1.0	13.6 ± 1.1	13.5 ± 0.6
B-8634	16.4 ± 1.6	15.0 ± 0.7	17.1 ± 0.7	15.4 ± 0.6	15.6 ± 0.9
Lactobacillus delbrueckii* subsp. *bulgaricus					
B-2455	14.6 ± 0.8	13.3 ± 0.6	10.3 ± 0.5	6.0 ± 0.5	6.3 ± 0.4
B-6543	20.1 ± 0.6	17.6 ± 0.7	17.9 ± 0.8	6.8 ± 0.2	14.9 ± 0.8
B-7638	19.8 ± 0.9	11.6 ± 0.6	12.5 ± 0.5	5.9 ± 0.4	9.1 ± 0.4
Streptococcus thermophiles					
B-2894	9.4 ± 0.4	10.3 ± 0.4	6.7 ± 0.1	10.1 ± 0.3	9.0 ± 0.4
B-3142	6.5 ± 0.6	8.1 ± 0.4	1.4 ± 0.4	7.8 ± 0.5	7.8 ± 0.4
B-3808	6.5 ± 0.5	7.1 ± 0.6	2.3 ± 0.5	2.4 ± 0.3	5.8 ± 0.5
B-5626	3.3 ± 0.4	8.1 ± 0.7	2.9 ± 0.5	2.4 ± 0.3	6.5 ± 0.6

It can be seen in Table 6 that the highest titratable acidity of the considered collection cultures was observed for *Lb. acidophilus* B-8634 (after 12 h – 98°T; after 24 h – 187°T), *Lb. delbrueckii* subsp. *bulgaricus* B-6543 (after 12 h – 96°T; after 24 h – 203°T), and *St. thermophiles* B-2894 (after 12 h – 94°T; after 24 h – 201°T).

High antagonistic activity with regard to pathogenic and commensal microorganisms is an obligatory condition for use of strain of lactic acid bacteria upon engineering of food additive. Activity of the considered strains was determined *in vitro* by deferred antagonism method on dense nutrient medium with regard to reference set of test strains of five pathogenic agents: *Shigella flexneri* 170, *Shigella sonnei* 863, *Escherichia coli* 157, *Staphylococcus aureus* 209, and *Salmonella enteridis* 36 (Table 7).

The experimental results demonstrated that among species of *Lactobacillus acidophilus* the strain B-8634 exhibited the highest antagonistic activity towards test cultures, herewith, the inhibition zone of *S. flexneri* was 16.4 mm, *S. sonnei* – 15.0 mm, *E. coli* – 17.1 mm, *S. aureus* – 15.4 mm, and *S. enteridis* – 15.6 mm. Among species of *Lactobacillus bulgaricus* the highest antagonistic activity towards test cultures was exhibited by the strain B-6543, herewith, inhibition zone of *S. flexneri* was 20.1 mm, *S. sonnei* – 17.6 mm, *E. coli* – 17.9 mm, *S. aureus* – 6.8 mm, and *S. enteridis* – 14.9 mm. Among species of *Streptococcus thermophiles* the highest antagonistic activity towards test cultures was exhibited by the strain B-2894, herewith, inhibition zone of *S. flexneri* was 9.4 mm, *S. sonnei* – 10.3 mm, *E. coli* – 6.7 mm, *S. aureus* – 10.1 mm, and *S. enteridis* – 9.0 mm.

Therefore, the performed studies made it possible to determine that high acid forming and antagonistic ability, resistance to phenol, bile and some antibiotics, as well as ability to ferment various carbohydrates were exhibited by the following collection strains: *Lactobacillus acidophilus* – strain B-8634, *Lb. delbrueckii* subsp. *bulgaricus* – strain B-6543, and *Streptococcus thermophiles* – strain B-2894. All their properties make it possible to propose these cultures as main components for engineering of three-strain additive with high probiotic activity.

Nutrient medium on the basis of soy milk (suspension) with addition of milk whey was used for submerged growing of the selected collection strain cultures. Regular nutrient medium for lactic acid microorganisms, fat free milk, was used as reference.

In order to evaluate optimum variant of nutrient medium experiments on cultivation of the selected strains with various composition were performed; the titer of grown microorganisms was estimated. The data are summarized in Table 8.

As can be seen in Table 8, optimum nutrient medium for cultivation is variant 5, where soy milk is used in combination with milk whey in amount of 30% of total medium bulk, since in variants 6 and 7 with lower content of soy milk the titer of microorganisms decreases. In variants 3 and 4 the titer of cells is slightly higher than in variant 5, and increase of content of soy milk increases the cost of nutrient medium.

Results of comparison of titer of lactic acid microorganisms on selected nutrient medium with reference are summarized in Table 9.

Table 8. Composition of nutrient media and titer of cells after their cultivation

Components of nutrient medium	**Variant of nutrient medium**						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Soy milk (suspension)	100	–	90	80	70	60	50
Milk whey	–	100	10	20	30	40	50
Amount of microorganisms							
Titer, CFU/ml	3.2×10^{9}	1.9×10^{7}	2.9×10^{9}	2.0×10^{9}	1.7×10^{9}	8.1×10^{8}	7.1×10^{8}

Table 9. Comparison of titer of cells after their cultivation on various nutrient media

Property	Variant of nutrient medium	
	soy milk (30%) + milk whey (70%)	Fat free milk
Titer, CFU/ml	1.7×10^{9}	1.1×10^{8}

The experimental results demonstrated that the most favorable nutrient medium for submerged cultivation of the selected collection strains was combined use of soy milk and milk whey, this variant was the most cost-efficient.

CONCLUSION

The experimental results demonstrated that among the 11 considered collection strains of lactic acid bacteria the following collection strains were characterized by high acid forming and antagonistic ability, resistance to phenol, bile and some antibiotics, as well as ability to ferment various carbohydrates: *Lactobacillus acidophilus* B-8634, *Lb. delbrueckii* subsp. *bulgaricus* B-6543, *Streptococcus thermophiles* B-2894. On the basis of selected lactic acid bacteria a liquid probiotic food additive was engineered and developed, which was the combination of these lactic acid strains grown on milk whey with soy milk containing at least 1.0×10^{9} CFU/ml of live microflora. The use of probiont strains in the additive increased inhibition zone of five pathogenic agents: *Shigella flexneri* 170, *Shigella sonnei* 863, *Escherichia coli* 157, *Staphylococcus aureus* 209 and *Salmonella enteridis* 36, which provided its high antibacterial properties. Due to resistance to bile and phenol the used culture will survive in poultry gastroenteric tract. The developed probiotic can be recommended for application in commercial poultry operation as innovative food additive providing sanitation and protection of diseases caused by violation of microbial balance in gastroenteric tract.

ACKNOWLEDGMENTS

The work was supported by the grant of the President of the Russian Federation for state support of young scientists MK-961.2017.11 (Contract No. 14.W01.17.961-MK).

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