# Formation of the bacterial community as the basis of probiotic supplement for livestock

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**Abstract.** The paper presents the results of research on the creation of a community based on lactobacilli and bacilli with antimicrobial activity against pathogens of intestinal infections in farm animals. *B. subtilis* strain MP 2 and *L. plantarum* strain MP 5 included in the consortium are characterized by resistance to artificial gastric juice and bile salts, as well as the ability to form extracellular hydrolytic enzymes (xylanase, carboxymethylcellulase, avicelase, β-glucosidase, amylase, phytase, protease and lipase), antimicrobial (acids, bacteriocins, siderophores and exopolysaccharides) and antioxidant metabolites. *B. subtilis* strain MP 2 and *L. plantarum* strain MP 5 did not have hemolytic activity and did not carry genes responsible for toxin production, which indicates the potential safety of this bacteria. It was concluded that the developed consortium is promising for use as a probiotic for animal husbandry.

## 1 Introduction

Probiotics are live microorganisms, the use of which has a positive effect on many functions of the host body; in particular, they are able to prevent the invasion of various pathogens of farm animals [1, 2]. The development of probiotics as an alternative to antibiotics is one of the approaches to combat the spread of various pathogens of intestinal infections [3, 4].

The effectiveness of probiotics is primarily determined by the microorganism strains they contain [5]. When selecting strains of microorganisms as potential probiotics, the following properties must be taken into account: they must have the ability to inhibit various pathogenic and opportunistic bacteria; characterized by colonization potential; adjust intestinal microbiota; have positive effects on the host's body; be non-toxic and non-pathogenic [6, 7].

To date, autochthonous lactic acid bacteria of the genus *Lactobacillus* and spore-forming bacteria of the genus *Bacillus* are widely used as probiotics for animal husbandry due to their ability to have a growth-promoting and preventive effect on animals [2, 4]. In recent years, probiotics based on various types of bacteria that can compensate, expand or enhance the functional properties of each other have attracted particular interest [8]. When developing these drugs, the key stage that determines their effectiveness is the creation of a bacterial community [3]. However, obtaining a consortium as the basis of a multispecies probiotic is

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a difficult task due to the fact that the bacteria included in the preparation with probiotic properties must be biocompatible.

The aim of this work was to create a bacterial community as the basis of a supplement with probiotic properties for the possibility of its use in animal husbandry.

## 2 Materials and methods

The work used strains MP 2, MP 4, MP 5, MP 14 and MP 23, which have antimicrobial potential against pathogens of intestinal infections in farm animals, from the collection of microorganisms of the Federal Center for Toxicological Radiation and Biological Safety (Kazan, Russia). Bacterial cultures were maintained by periodic subcultures at 4°C on LB (Luria Bertani) (Condalab, Spain) and MRS (DeMan, Rogosa and Sharpe) (Conda, Spain) agar media.

The study of the biocompatibility of bacterial strains was carried out using the method of direct co-cultivation of the latter on the surface of various solid nutrient media (drip technique) [9]. The peculiarities of interactions between bacterial strains were determined visually by the nature of the growth and development of their colonies: type 1 - antagonism: the suppressive influence of the inoculated bacterial culture on the growth of the studied strain; type 2 - neutrality: independent growth of the culture under study; type 3 - contact progression: stimulating effect of the studied strain by inoculating a bacterial culture.

To more accurately assess the characteristics of the interaction of selected bacterial strains (bacillus and lactobacilli), the dynamics of their growth was studied on a nutrient medium of the following composition (g/l): glucose - 21.0; meat-peptone broth - 20.0; yeast extract - 5.0; peptone - 2.0; sodium acetate - 4.0; ammonium sulfate - 2.0; potassium phosphate - 1.5; magnesium sulfate - 0.1; manganese sulfate - 0.05 (pH 6.9). Bacteria were grown for 12 h at 37 °C, 50 rpm with aeration. Every 2 h, the culture liquid was taken to count the number of vegetative cells per unit volume (CFU/ml).

Determination of the generic affiliation of the selected bacteria was carried out according to their morphological, cultural and physiological-biochemical characteristics [10, 11].

The ability of selected bacterial strains to grow at different temperatures was determined by growing them in medium LB (for bacillus) and MRS (for lactobacilli) at (15±1) and (45±1)°C for 24 h [12]. The ability of bacterial strains to grow under conditions of different salinity concentrations was assessed by incubating them in the above agar media containing 4 and 6.5% sodium chloride at 37°C for 24 h [11].

The catalase activity of selected bacteria was assessed on the surface of an agar nutrient medium with bacterial colonies grown on it by applying a 3% hydrogen peroxide solution in a volume of 5 µl [13]. The formation of oxygen bubbles was used to judge the ability of the studied strains to produce the enzyme catalase.

The species identity of the selected bacterial strains was confirmed by molecular genetic analysis [11, 14].

The resistance of selected bacteria to artificial gastric juice and bile salts was studied *in vitro* using the method described in [15, 16]. To do this, vegetative bacterial cells were incubated in MRS broth containing pepsin (3 mg/ml, pH 3.0) or bile (0.3%) at 37°C for 2 or 8 hours. Initial titer of viable cells for the studied cells bacteria was $8.91 \times 10^7$ CFU/ml (control). The number of bacterial cells (CFU/ml) of the strains was determined by the standard plate method. The above nutrient media were used as a medium for counting the number of viable bacterial cells.

The adhesive ability of selected bacterial strains was assessed *in vitro* using human colorectal adenocarcinoma HT-29 cells [16, 17].

The hemolyzing activity of selected bacteria was determined using Columbia blood agar containing 5% (w/v) sheep blood [17, 18].

The search for genes encoding various toxins in selected bacterial strains was carried out using polymerase chain reaction (PCR) with specific oligonucleotide primers given in [17, 19-21]. PCR products were fractionated on 1.5% agarose gel (Bio-Rad) and visualized under ultraviolet light.

The substrate-hydrolyzing ability of the enzymes of selected bacteria was assessed using a modified MRS agar medium (for lactobacilli) and a synthetic medium (for bacillus) containing (g/l) sodium citrate - 1.29, $K_2HPO_4$ - 9.6, $(NH_4)_2HPO_4$ - 4.75 and $MgSO_4x7H_2O$ - 0,18 (pH 7.0) [22]. Xylan, sodium carboxymethylcellulose, starch, pectin, casein, sodium phytate, olive oil, Tweens 20, 40, 60 and 80 were used as inducers of enzyme production. Bacteria were cultivated at temperature of 37 °C. The ability of the strain to produce enzymes was determined by the formation of zones of substrate hydrolysis around bacterial colonies.

For deep cultivation of selected bacterial strains to assess their ability to produce extracellular hydrolytic enzymes, liquid media containing inductors of their synthesis corresponding to enzymes as food sources were used [10, 23, 24]. The activity of bacterial hydrolases was assessed using methods described earlier by us [10] and in works [23-26].

The ability of selected bacterial strains to form siderophores was determined on a differential agar medium containing the dye chromasurol S (CAS medium) [27]. Cultivation of the strains was carried out at a temperature of 35 °C for 5 days. The appearance of clearing zones on CAS agar was used to judge the ability of bacteria to secrete siderophores.

The ability of selected bacteria to produce bacteriocins was assessed using the disk diffusion method [28].

The ability of selected bacterial strains to form exopolysaccharides was determined on an agar medium containing ruthenium red dye (Sigma, USA) [29]. Cultivation of bacterial strains was carried out at a temperature of 35°C until changes in the color of their colonies appeared. The absence of changes in the color of the colonies indicated the ability of bacteria to form exopolysaccharides.

The ability of selected bacteria to produce biologically active metabolites with antiradical potential was assessed by the free radical quenching method and the FRAP method using 2,2-di-phenyl-1-picrylhydrazyl (Alfa Aesar, USA) and potassium ferricyanide (Sisco Research Laboratories Pvt. Ltd., India) reagents, respectively [30]. The experiment used the culture liquid of the strains obtained after their cultivation in nutrient broths at a temperature of 37°C and 50 rpm.

Statistical analysis was carried out using Microsoft Excel. To describe and compare characteristics, construction of 95% confidence intervals for means was used.

## 3 Results and discussion

During the cultivation process, different types of microorganisms with probiotic properties are able to enter into various types of relationships with each other [3, 9]; therefore, the creation of a bacterial association as the basis of a multispecies probiotic is a difficult task. In order to study the biocompatibility of bacterial strains, we used the method of co-cultivating the latter (drip technique) on the surface of various agar nutrient media.

An assessment of the biocompatibility of bacterial strains characterized by antimicrobial potential against pathogens of intestinal infections of farm animals showed that most of them have an antagonistic effect on each other (Table 1).

Based on the results of their ability to suppress the growth of pathogens of intestinal infections, as well as biocompatibility, the most promising bacteria were selected, namely strains MP 2 and MP 5.

In order to more accurately determine the characteristics of the interaction between the strains we selected, we assessed the dynamics of the growth of their bacterial cells on a liquid

modified MRS medium, which allows for the co-cultivation of various types of lactic acid and spore-forming bacteria.

**Table 1.** Results of determining the types of interactions between bacterial strains with antimicrobial potential against pathogens of intestinal infections of farm animals[a].

| | **MP 2** | **MP 4** | **MP 5** | **MP 14** | **MP 23** |
|---|---|---|---|---|---|
| MP 2 | | 1 | 3 | 2 | 3 |
| MP 4 | 1 | | 1 | 1 | 2 |
| MP 5 | 3 | 2 | | 1 | 1 |
| MP 14 | 1 | 1 | 1 | | 2 |
| MP 23 | 2 | 1 | 1 | 1 | |

[a] Type 1 - antagonism, type 2 - neutrality, type 3 - contact progression.

As a result of joint deep cultivation of bacterial strains MP 2 and MP 5, their significant influence on each other's development was shown. Co-cultivation of the selected strains for 10 hours of growth led to an increase ($p<0.05$) in the number of MP 2 and MP 5 cells, respectively, by 1.59 ($1.15 \times 10^9$ CFU/ml) and 2.37 ($1.80 \times 10^9$ CFU/ml) times relative to the bacterial content in monocultures ($7.24 \times 108$ and $7.59 \times 10^8$ CFU/ml, respectively) (Figure 1).

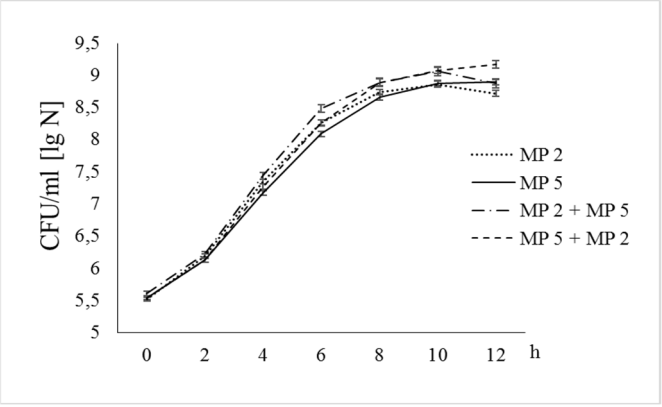


**Figure 1**. Change in the number of bacterial cells (CFU/ml) of MP 2 and MP 5 strains during their co-cultivation in liquid modified MRS medium (differences between the values of the cell content of MP 2 strain and MP 2 strain under conditions of its co-growth with MP 5 strain on 6-12 hours of cultivation are statistically significant at $p=0.05$; cell contents of MP 5 strain and MP 5 strain under conditions of joint growth with strain MP 2 at 6-12 hours of cultivation are statistically significant at $p=0.05$)[a]. [a] MP 2 + MP 5 - the number of bacterial cells of MP 2 strain when it grows together with MP 5 strain; MP 5 + MP 2 – the number of bacterial cells of MP 5 strain during its joint growth with MP 2 strain.

The possibility of using selected bacteria as a promising basis for probiotic supplements for industrial animal husbandry requires their identification.

According to the study of physiological and biochemical properties, MP 2 and MP 5 strains were assigned to the genera *Bacillus* and *Lactobacillus*, respectively (Table 2).

The species of bacteria was confirmed using molecular genetic analysis.

**Table 2.** Basic physiological and biochemical properties of selected bacteria[a].

| Parameters | MP 2 | MP 5 |
|---|---|---|
| Growth at 15 $^0$C | + | + |
| Growth at 45 $^0$C | + | - |
| Cultivation in presence 4,0 % NaCl | + | + |
| Cultivation in presence 6,5 % NaCl | + | + |
| Formation of gas from glucose | - | + |
| Production of extracellular catalase | + | + |

[a] (-) – absence, (+) – presence of a sign.

Identification of nucleotide sequences of fragments using an international database made it possible to determine the 99-100 % degree of similarity of the obtained sequences in bacteria with those found in NCBI (Table 3).

**Table 3.** Comparison of sequenced nucleotide sequences of the 16SpDNA fragment of selected bacteria with those deposited in the international database.

| Identifiable strain | Strain in the database | % identity |
|---|---|---|
| MP 2 | *B. subtilis, strain Lactipan,* number: AJ277905.1 | 100 |
| | *B. subtilis, strain J2,* number: MG575432.1 | 99 |
| | *B. subtilis, strain AU-2,* number: MF538574.1 | 99 |
| MP 5 | *L. plantarum*, *strain G8,* number: JX183220.1 | 99 |
| | *L. plantarum, strain LP*, number: KJ802485.1 | 99 |
| | *L. plantarum, strain BS404,* number: MF521893.1 | 99 |

Thus, analysis of the nucleotide sequence of the 16SrDNA region, taking into account the morphological characteristics of the bacteria, made it possible to establish that MP 2 and MP 5 strains belong, respectively, to the species *Bacillus subtilis* and *Lactobacillus plantarum*.

Some strains of *Bacillus subtilis* are capable of stimulating the growth of *Lactobacillus plantarum*. [31, 32]. Information began to appear abroad regarding the ability of metabolites formed by bacilli, including cyclic dipeptides [33], surface proteins [34], subtilisin [35], enzymes (catalase, superoxide dismutase, amylase, protease) [34-36], to stimulate the development of test-cultures of lactic acid microorganisms under model conditions.

One of the main factors that limits the inclusion of bacterial strains with probiotic potential in multispecies preparations is the resistance of the latter to stressful conditions of the digestive system [2, 37]. The transit of probiotic strains through the gastrointestinal tract of animals is associated with exposure to such major stressors as gastric hydrochloric acid and bile salts entering the intestine, which can significantly damage bacterial cells and reduce their survival.

Establishing the resistance of bacterial strains to artificial gastric juice and bile salts showed their ability to maintain viability (Table 4).

The main property that characterizes microorganisms with probiotic properties is their colonizing ability [6, 16, 17], which determines adhesion to the epithelial cells of the animal's intestine. To assess the colonizing properties of the strains, we assessed their adhesive ability to human intestine epithelial cells.

An assessment of the adhesive properties of the selected bacteria showed that the studied strains were characterized by the ability to adhere to HT-29 cells. After 2 hours of incubation, 5.84 ± 0.26 and 6.51 ± 0.33 log CFU/well of bacterial cells adhered for *B. subtilis* strain MP

2 and *L. plantarum* strain MP 5, respectively (Table 4), indicating sufficient ability of the latter to colonize intestinal epithelial cells.

**Table 4.** Probiotic properties of selected bacterial strains.

| | ***B. subtilis* strain MP 2** | ***L. plantarum* strain MP 5** |
|---|---|---|
| Resistance to effects of artificial gastric juice and bile salts Content of bacterial cells, CFU/mL [lg N] | | |
| at the beginning of the experiment | 7.95 ± 0.24 | 7.95 ± 0.36 |
| pepsin, pH 3.0, 2 h | 7,26 ± 0.18 | 7,83 ± 0.22 |
| 0.3 % oxgall, 8 h | 7,92 ± 0,20 | 7,99 ± 0.27 |
| Adhesion to HT-29 cells CFU/well of bacterial cells [lg N] | | |
| at the beginning of the experiment | 7.90 ± 0.26 | 7.90 ± 0.33 |
| 37 °C, 2 h | 5.84 ± 0.26 | 6.51 ± 0.33 |
| Hemolysis | | |
| | - | - |
| Toxin-related genes | | |
| hbl, nhe, cyl, cytK, selk, selq, gelE | - | - |

*B. subtilis* strain MP 2 and *L. plantarum* strain MP 5 did not have hemolytic activity and did not carry toxin-related genes (Table 4), which indicates the potential safety of the studied bacteria.

Recently, special attention has been attracted to preparations with probiotic properties, which contain various types of bacteria with complex properties [3, 8]. One of the manifestations of the probiotic functions of some strains of spore-forming and lactic acid bacteria is the ability of the latter to form hydrolytic enzymes, which can play a significant role in the digestion process in farm animals by improving the digestibility of the components of their diet [2, 6, 38]. Table 5 presents the results of assessing the ability of *B. subtilis* strain MP 2 and *L. plantarum* strain MP 5 to produce extracellular hydrolases.

**Table 5.** Ability of selected bacterial strains to form extracellular hydrolytic enzymes [a].

| | **B. subtilis strain MP 2** | **L. plantarum strain MP 5** |
|---|---|---|
| Xylanase | + | - |
| Cellulase | + | - |
| Amylase | + | + |
| Pectinase | - | ± |
| Phytase | + | + |
| Protease | + | + |
| Lipase | + | + |

[a] (-) – absence, (+) – presence of a sign.

It was found that the *L. plantarum* strain MP 5 did not break down polysaccharides such as xylan and cellulose. The most active of the strains we selected was *B. subtilis* strain MP 2, whose enzymes simultaneously hydrolyzed most substrates. Under the conditions used in our study, both strains had no or weak ability to secrete extracellular enzymes of the pectinase complex.

This work established the ability of *B. subtilis* strain MP 2 and *L. plantarum* strain MP 5 to grow on liquid nutrient media containing wheat and soy flour, which are the main components of feed for farm animals (results not shown).

The study of hydrolase activity in the cultural supernatant of selected bacteria obtained through homogeneous submerged cultivation confirmed significant strain differences in the ability to produce extracellular enzymes (Figure 2).

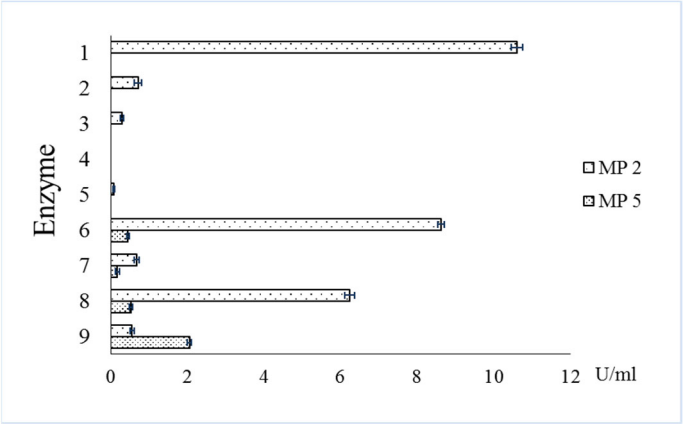


**Fig. 2**. Maximum activity of hydrolytic enzymes of selected bacterial strains during deep cultivation: 1 - xylanase, 2 - carboxymethylcellulase, 3 - avicelase, 4 - β-glucosidase, 5 - cellulase relative to filter paper, 6 - amylase, 7 - phytase, 8 - protease, 9 – lipase.

Among the bacteria studied, the bacillary strain MP 2 had the highest activity of amylase, phytase and protease (8.63±0.15, 0.68±0.17 and 6.24±0.12 U/ml, respectively), which is 19.2, 2.6 and 11.8 times higher ($p<0.05$) relative to the level of production of these enzymes in the lactobacillary strain MP 5. However, strain MP 5 was distinguished by a higher level of production compared to strain MP 2 lipase (2.06±0.05 U/ml), the activity of which was 3.7 times greater ($p<0.05$). Under the conditions used in our study, strain MP 5 was not characterized by the ability to secrete extracellular enzymes of the cellulase complex.

The production of extracellular hydrolases by representatives of the genus *Bacillus* and *Lactobacillus* is mainly determined by cultivation conditions (acidity, temperature and aeration), the composition of the nutrient medium used for cultivation (nutrient sources), the growth phase and the characteristics of their genome, which determine strain-specific characteristics [2, 10, 38-40].

In order to create a drug with antimicrobial, antioxidant and immunomodulatory properties for animal husbandry, the ability of selected strains to form acids, siderophores, exopolysaccharides and bacteriocins was determined (Table 6).

**Table 6.** Ability of selected bacterial strains to form antimicrobial and antioxidant compounds[a].

| | **B. subtilis strain MP 2** | **L. plantarum strain MP 5** |
|---|---|---|
| | **Antimicrobial compounds** | |
| Acids | - | + |
| Siderophores | + | - |
| Bacteriocins | + | + |
| Exopolysaccharides | + | + |
| | Antioxidant compounds | |
| | + | + |

[a] (-) – absence, (+) – presence of a sign.

Of the 5 biologically active compounds analyzed, *L. plantarum* strain MP 5 produced acids, bacteriocins, exopolysaccharides and metabolites with antioxidant potential. In terms of the production of antimicrobial compounds, the bacillary strain MP 2 turned out to be the most active. This bacterium was capable of producing siderophores, bacteriocins and exopolysaccharides.

## 4 Conclusion

As a result of the research, an association was created based on the strains *B. subtilis* strain MP 2 and *L. plantarum* strain MP 5, which has antimicrobial potential against pathogens of intestinal infections of farm animals. The bacterial strains included in the community we developed are characterized by resistance to bile and pepsin, the ability to form extracellular hydrolytic enzymes (xylanase, carboxymethylcellulase, avicelase, β-glucosidase, amylase, phytase, protease and lipase), antimicrobial (acids, bacteriocins, siderophores and exopolysaccharides) and antioxidant metabolites, which determines their prospects for use as the basis for additives with probiotic properties in animal husbandry.

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