Antagonistic properties and biocompatibility as important principles for development of effective and biosafety probiotic drugs

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Abstract. Antagonistic properties and peculiarities of inter-strain interactions of probiotic microorganisms were investigated. The study of the antagonistic activity of probiotic strains was carried out by the methods of perpendicular strokes (or delayed antagonism) and the diffusion method (block modification) in relation to opportunistic and pathogenic microorganisms. Most probiotic strains had moderate to high antagonistic activity against both gram-positive and gram-negative pathogens. The results obtained allowed us to select the most active strains, namely L. plantarum AS-41, L. acidophilus A-14, B. subtilis S-2 and B. subtilis S-18, which have a high probiotic potential. To assess biocompatibility, the method of cocultivation of the selected strains on a modified agar MRS medium was used. The strains that showed biocompatibility of the type "mutual neutrality" or "contact progression" were selected to create a consortium of probiotic microorganisms. Further study of the investigated strains of probiotic bacteria opens up the possibilities of their use for the manufacture of a combined preparation for veterinary purposes, for the prevention and treatment of diseases of the gastrointestinal tract of farm animals.

1. Introduction

The use and implementation of the genetic potential of farm animals, contributing to obtaining the maximum values of economic indicators in animal husbandry, taking into account the country's biosafety, possibly with the necessary quality of their feeding, strict adherence to a set of veterinary and sanitary rules and scientific programs for the use of medicinal products [1, 2]. Nevertheless, in the conditions of agro-industrial complexes, as a result of poor quality of feed, unbalanced diets, unsatisfactory conditions for keeping animals and massive uncontrolled use of antibiotics, there is a decrease in their adaptive capabilities, which leads to the development of diseases associated with a violation of the microecology of the digestive tract [3-6].

Gastrointestinal diseases of farm animals, especially young animals, are an urgent problem of veterinary medicine, which attracts the interest of researchers and veterinarians, who daily encounter microecological disorders in clinical pathological conditions of various origins [7, 8]. With
gastrointestinal diseases caused by pathogenic microorganisms, many manifestations of disorders in the functioning of organs and systems of the macroorganism are associated.

Currently, for the prevention and treatment of gastrointestinal disorders of farm animals, a number of measures are being taken to stimulate their natural resistance [1, 9]. Among the methods used to correct dysbacteriosis of the gastrointestinal tract of the body, probiotic drugs and their waste products play a key role in connection with the positive effects that are observed both objectively and subjectively [1, 8-11]. The use of probiotics and prebiotics in animal husbandry promotes the colonization of the intestine with endogenous microflora, which suppresses opportunistic and pathogenic microorganisms, enhances the absorption of nutrients and activates the defenses of the macroorganism. The emergence of new generation products, which include not only various active probiotic bacteria, but also their waste products, is associated with the improvement of technologies for their production on an industrial scale and the observed low or no negative effects from their use [11, 12].

Microorganisms that exhibit probiotic activity include strains of lactobacilli, spore aerobic bacilli, propionic acid bacteria, yeast fungi, enterococci, thermophilic streptococci, indigenous Escherichia [5, 7-10, 12-16]. Nevertheless, numerous studies of biotechnologists show that the most promising for the creation of complex probiotics are representatives of the normal flora of the digestive tract of a macroorganism, which have minimal side effects with their long-term use [10]. Currently, the most common types of microorganisms used for the manufacture of probiotic preparations are *Bacillus subtilis*, *B. licheniformis*, *B. cereus*, *Bifidobacterium adolescentis*, *B. bifidum*, *B. breve*, *B. infantis*, *B. longum*, *Enterococcus faecalis*, *E. faecium*, *Escherichia coli*, *Lactobacillus acidophilus*, *L. casei*, *L. delbrueckii*, *L. helveticus*, *L. fermentum*, *L. rhamnosus*, *L. salivarius*, *L. plantarum*, *Propionibacterium acnes*, *P. freudenreichi*, *Streptococcus cremoris*, *S. lactis*, *S. salivarius*, *Clostridium butyricum*, *Saccharomyces boulardii*, and many others [1, 2, 8, 10, 12]. It should be emphasized that all probiotic microorganisms used in biotechnology have distinct cultural-morphological, physiological-biochemical and biotechnological properties. The use of new probiotic strains in biotechnology for the industrial production of biological products becomes permissible after a detailed study of their biological activity, which must comply with the requirements established by regulatory documents [10-12].

The most important properties that determine the applicability of probiotic microorganisms are their antagonistic properties and biocompatibility [11, 17]. The antagonistic properties of probiotic microorganisms are due to the formation of various organic acids (lactic, propionic and acetic), alcohols, hydrogen peroxide, as well as the production of lysozyme and bacteriocins of a wide spectrum of action [1, 2, 8, 9, 12, 18]. A number of authors point out that it is the ability of probiotic microorganisms to form organic acids from carbohydrates that leads to a decrease in the pH level in the medium and, thereby, inhibition of the growth of other types of microorganisms [2, 9, 12, 18, 19]. In addition, they can inhibit the development of microorganisms due to faster reproduction or a short lag phase. The process of biosynthesis of specific antibacterial proteins, bacteriocins, is controlled by intercellular communication ("quorum sense") and is a mechanism that allows you to change the density of populations of microorganisms [20].

It is preferable in the development of probiotic formulations to mix different species and genera of antagonistically active isolates of microorganisms. In comparison with monocultures, microbial consortia are able to use complex organic substances and substrates of heterogeneous composition as a source of nutrition. The consortium of microorganisms, consisting of various active strains, has an increased resistance to the effects of a variety of adverse environmental factors and toxic compounds, as well as increased productivity [2, 21].

Developers of monocomponent probiotics do not face the problem of crop biocompatibility. The nature of interspecific interactions is not always taken into account when developing multicomponent preparations [17], especially when the technology is used to mix dried individual monocultures. This method of obtaining drugs does not allow further predicting the behavior of probiotic cultures in the host's body. The creators of liquid multistain probiotics at the stage of drug design, first of all, face
the problem of selecting strains studied for biocompatibility within a multicomponent association during cultivation.

The aim of the study was to establish the antagonistic properties and biocompatibility of new strains of probiotic microorganisms.

2. Materials and methods

The objects of our research were probiotic strains of \textit{L. plantarum} P-27, \textit{L. plantarum} AS-41, \textit{L. fermentum} F-10, \textit{L. acidophilus} A-14 and \textit{B. subtilis} S-2, which were obtained from the collection of bacterial cultures of the Department of Biotechnology of Federal Center for Toxicological, Radiation and Biological Safety (FSBSI "FCTRBS-RRVI"). The following test strains were used as indicator cultures: \textit{Staphylococcus aureus}, \textit{Salmonella typhimurium} and \textit{Escherichia coli}. Cultures of bacterial isolates were maintained by the method of periodic subcultures at a temperature of 4 °C on agar media of MRS (HiMedia Laboratories, India) and GRM (FBUN GNTs PMB, Russia).

The study of the antagonistic activity of probiotic strains was carried out using the methods of perpendicular strokes (or delayed antagonism) and the diffusion method (block modification) [22, 23].

To do this, a strip of lactobacilli or bacilli culture was applied to the bottom of a dish with a dense nutrient medium using a loop with a diameter of 2 mm. The strains were incubated at 37 °C for 72 hours.

After the growth and formation of bacterial antibiotic substances diffusing into the thickness of the nutrient agar, pathogenic cultures were inoculated. To determine the antagonistic activity of the microorganism, test cultures, previously grown in the infusion broth of the heart of the brain (HBI broth) for 18 hours, were inoculated to the grown culture. The inoculation of the test strains was carried out using a bacteriological loop (diameter 1 mm) in the direction from the growth zone of the probiotic strain, without touching it, and perpendicular to it. The results were recorded after 24 h of incubation at 37 °C according to the size of the zone of no growth of the test culture. The growth control of the test culture was their inoculation on plates with the same medium without the test culture. The results were recorded by inhibition of the growth of test strains sensitive to antagonistic effects: up to 2 mm – 1 point, from 3 to 6 mm – 2 points, 7-14 mm – 3 points, more than 15 mm – 4 points.

In block method, the studied probiotic strains were plated in Petri dishes by the deep method in agar medium MRS Agar or GRM-agar and incubated at a temperature of 30 °C for 24 hours. This manipulation was performed to accumulate inhibitory compounds in agar. Then, an agar block with a germinated bacterial culture was excised using a sterile cork drill and placed in another Petri dish on the surface of a new agar medium seeded with a continuous culture of the test strain. Then this cup was kept for 1 hour in the refrigerator to diffuse the active inhibitory compounds from the block into the thickness of the agar and to prevent premature growth of the test culture. Then incubation was carried out at a temperature of 37 °C for 24 hours. The degree of antagonistic activity of the tested bacteria was judged by the size of the zone of inhibition of growth of the test culture around the agar block.

To study the biocompatibility, the probiotic strains were grown on a dense modified MRS medium of the following composition (g/L): NaCl - 3.0, KH2PO4 - 1.0, MgSO4 · 7H2O - 0.05, MnCl4 · 4H2O - 0.03, HOOCC (OH) (CH2COONH4) 2 - 1.3, D-glucose - 13.3, peptone - 8.0, yeast extract - 3.3, pancreatic hydrolyzate of fishmeal - 17.0. To obtain a dense nutrient medium, 1.3% agar was added to the above broth. Served as control inoculations of the investigated cultures of microorganisms. The culture dishes were incubated in a thermostat at 37 ± 0.5 °C for 48 h. To establish the sterility of the nutrient agar used, sterile Petri dishes were filled with the same nutrient agar (negative control).

The nature of interactions of probiotic strains in conditions of co-cultivation \textit{in vitro} on solid nutrient medium was assessed by the growth and development of their colonies using the following types [24]:

1. - contact regression: inhibition of the growth of the investigated probiotic strain by the sown crop;
2. - neutrality: independent growth of the studied and sown strains;
3. - contact progression: stimulating the growth of probiotic cultures of each other;
4. - antagonism: the presence of growth retardation of the sown strain.

The results of the antagonistic action of probiotic microorganisms, detected by the method of agar blocks, were estimated by the size of the zone of inhibition of the growth of the test strain around the agar block [23].

The experiments were carried out in 3 biological and 3 analytical replicates [25-27]. Statistical processing of the results was carried out by finding the arithmetic mean values and their standard errors using the standard software package Microsoft Office Excel 2013. The significance of differences was evaluated using the Student t-test, the differences were considered significant at p<0.05.

3. Results and discussion

One of the important properties that active probiotic strains should be characterized by is their pronounced antagonistic activity against a wide range of opportunistic and pathogenic microorganisms.

The study of the antagonistic activity of the studied strains of lactobacilli and bacilli by the method of perpendicular strokes and the diffuse method showed that almost all of them have an inhibitory effect on indicator cultures; nevertheless, some had a pronounced sensitivity to test-isolates. The results of the study of the antagonistic properties of the studied bacterial strains in relation to opportunistic and pathogenic microorganisms are presented in table 1 and figure 1.

Table 1. Antagonistic activity of probiotic strains against gastrointestinal pathogens (method is perpendicular strokes)a.

| Probiotic strains | *Staphylococcus aureus* | *Salmonella typhimurium* | *Escherichia coli* |
|-------------------|------------------------|-------------------------|------------------|
| L. plantarum P-27 | -                      | ++                      | +++              |
| L. plantarum AS-41 | +++                    | +++                     | ++++             |
| L. fermentum F-10  | +++                    | -                       | +++              |
| L. fermentum F-31  | -                      | -                       | ++++             |
| L. acidophilus A-14 | ++++                   | +++                     | +++              |
| L. acidophilus A-58 | ++++                   | +++                     | -                |
| B. subtilis S-2    | +++                    | +++                     | ++++             |
| B. subtilis S-18   | +++                    | +++                     | +++              |

*a++++* growth inhibition zone above 15 mm, +++ growth inhibition zone 7-14 mm, ++ growth inhibition zone 3-6 mm, + growth inhibition zone up to 2 mm, - no antagonistic activity was found.

As can be seen from table 1, most probiotic strains had moderate to high antagonistic activity against both gram-positive and gram-negative pathogens. Strains of *L. plantarum AS-41*, *L. acidophilus A-14*, *B. subtilis S-2* and *B. subtilis S-18* were the most active against all tested pathogenic strains. The greatest antagonistic activity of *L. acidophilus A-14*, *L. acidophilus A-58*, *B. subtilis S-2*
and B. subtilis S-18 strains against Staphylococcus aureus, and L. plantarum AS-41, L. fermentum F-31, B. subtilis S-2 and B. subtilis S-18 in relation to E. coli was recorded. The probiotic strains of L. plantarum AS-41, L. acidophilus A-14, L. acidophilus A-58, B. subtilis S-2 and B. subtilis S-18 showed the same antagonistic activity against Salmonella typhimurium, while the isolates of L. fermentum F-10 and L. fermentum F-31 were not able to inhibit the growth of this test culture.

Similar results on antagonistic activity were obtained using the diffuse method (figure 1).

**Figure 1.** Antagonistic activity of probiotic strains against gastrointestinal pathogens (diffusion method).

Among the strains of lactobacilli, L. plantarum AS-41 and L. acidophilus A-14 also had the highest antagonistic activity. Depending on the test object, the growth retardation zone averaged 7–20 mm. Strains of spore-forming bacteria Bacillus subtilis showed a high level of antagonistic activity against all studied opportunistic and pathogenic microorganisms, the growth inhibition zone averaged 12–28 mm.

Differences in the antagonistic activity of probiotic strains in relation to opportunistic and pathogenic microorganisms are explained by the variable composition of the bacterial cell peptidoglycan, which is a regulator of relationships in the "prokaryote - prokaryote" system, as well as the specificity of the action of metabolites of lactobacilli and bacilli on opportunistic and pathogenic microorganisms [9]. The metabolic products of probiotic microorganisms contain a huge amount of various bacteriocins and enzymes, including lysozyme. For example, isolates of B. subtilis and other probiotic strains contain up to 200 antibiotic substances that can affect the surrounding microflora [28, 29].

As a result of the first series of experiments, we selected strains of microorganisms L. plantarum AS-41, L. acidophilus A-14, B. subtilis S-2 and B. subtilis S-18 to assess their biocompatibility.

When developing a probiotic preparation containing an association of active forms of living bacteria and their metabolic products, the main criterion for assessing its applicability is to identify a stable consortium that excludes antagonism between species. With the joint incubation of biocompatible strains of probiotics, the task of developing a consortium is achieved, which has an enhanced set of necessary biotechnological properties inherent in individual microorganisms.
For the study, we used a method for determining the types of interactions between probiotic microorganisms, based on the analysis of the nature of growth of bacterial colonies during their joint growth on a medium, as well as the method of agar blocks, which is based on the ability of metabolites of microorganism cultures to penetrate into the agar and inhibit the growth of the test object located in the diffusion zone.

In the course of studying the relationship of probiotic strains of microorganisms in conditions of co-cultivation in vitro, two main types of interaction were revealed: "neutrality" (2) and "contact progression" (3). The results of evaluating the interaction between strains are presented in table 2. The absence of zones of growth retardation of probiotic bacteria during their joint cultivation on a dense modified MRS medium indicated their biocompatibility.

**Table 2.** Results of interaction of probiotic strains of microorganisms.

|               | L. plantarum AS-41 | L. acidophilus A-14 | B. subtilis S-2 | B. subtilis S-18 |
|---------------|---------------------|---------------------|----------------|-----------------|
| L. plantarum AS-41 | 4                   | 3                   | 2              |                 |
| L. acidophilus A-14 | 4                   | 3                   | 2              |                 |
| B. subtilis S-2   | 3                   | 2                   |                 |                 |
| B. subtilis S-18   | 2                   | 2                   |                 |                 |

As can be seen from the table, during the joint cultivation of L. plantarum AS-41 and B. subtilis S-2 strains, an interaction of the "contact progression" type is observed: probiotic microorganisms and their metabolic products have a stimulating effect on the growth of each other (mutualism, synergism, satelitism).

There is a form of symbiosis between probiotic microorganisms, in which they are able to develop in nutrient media, mutually complementing each other's needs [17]. Most likely, the accumulation of some strains of various organic substances contributes to the formation of compounds by other strains, which ultimately can lead to an increase in the antimicrobial properties of the consortium in relation to pathogenic microflora.

The study of the interaction of the probiotic strains under study using the agar block method also showed that most cultures did not have an antagonistic effect on the growth of each other (table 3).

**Table 3.** Assessment of the antagonistic activity of probiotic strains.a.

|               | L. plantarum AS-41 | L. acidophilus A-14 | B. subtilis S-2 | B. subtilis S-18 |
|---------------|---------------------|---------------------|----------------|-----------------|
| L. plantarum AS-41 | pgr                 |ngr                  |                |                 |
| L. acidophilus A-14 | pgr                 |ngr                  |                |                 |
| B. subtilis S-2   |ngr                  |ngr                  | pgr            |                 |
| B. subtilis S-18   |ngr                  |ngr                  | pgr            |                 |

angr - no growth retardation, pgr - presence of growth retardation.

Factors and mechanisms of microbial regulation of the antagonistic properties of probiotic bacteria are diverse: an increase in the antagonistic activity of a culture may be due to conditions for improving its metabolic and growth parameters [20], the effect of certain inductors [30-32]; inhibition of this property can be caused by inactivation of antimicrobial factors, a negative effect on the gene activity of metabolic products [33], on the metabolism of the antagonist of the regulatory bacterium. If we consider the microbial regulation of intrageneric antagonism, which is most likely associated with bacteriocins, the inhibition of the trait is explained by the interfering effect of phospholipids contained in membranes [34] or the action of proteolytic enzymes of regulatory bacteria that destroy antibiotic compounds [35] or inducers of their biosynthesis [36, 37]. An increase in closely related antagonism
caused by the effect of metabolic products of microorganisms is explained by the cross influence of quorum molecules that mediate the manifestation of bacteriocinogenesis [30, 32, 38, 39].

The obtained results of studying the intergeneric synergism of the selected probiotic strains give grounds to assert the possibility of their joint cultivation, as well as a higher antagonistic potential and stability during storage of a consortium of cultures, which makes it possible to solve the complex biotechnological problem of developing probiotics containing an association of active forms of living bacteria and their metabolic products.

4. Conclusions
The results of the study of antagonistic activity by the method of perpendicular strokes and the diffuse method significantly showed differences in the ability of the studied strains to suppress the growth and reproduction of opportunistic and pathogenic microorganisms. Certain probiotic strains of lactobacilli and bacilli had a pronounced sensitivity to test-isolates. As a result of the research, taking into account the antagonistic properties of the probiotic microorganisms under study, we have selected biocompatible strains: L. plantarum AS-41 and B. subtilis S-2. Further study of probiotic strains opens up the possibility of their use for the manufacture of a combined preparation for veterinary purposes, for the prevention and treatment of diseases of the gastrointestinal tract of farm animals.

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