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ORIGINAL ARTICLE

Antimicrobial action of a bacterial consortium containing strains of the genus *Bacillus*

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Multicomponent biopreparations containing several symbiotic strains of microorganisms are gaining increasing popularity due to their heightened efficiency and improved biotechnological properties. In this connection, the purpose of this research was to study the antimicrobial action of a bacterial consortium consisting of 2 strains of *Bacillus subtilis*, as well as natural strains of the genus *Bacillus*. Strain of *Escherichia coli* was used as a test culture. We used methods of delayed (perpendicular bands, agar blocks, agar wells) and direct (co-incubating in a liquid medium) antagonism to achieve this aim. As a result, the antagonistic effect of the microbial composition against *Escherichia coli* was improved in compared to the single action of each strains included in the consortium. In agar media, the largest increase of the radius of zone of growth inhibition was noted from 6.65 mm to 10.67 mm, i.e. by 60.5%, and in liquid media – an improvement in the growth blocking index from 0.188 to 0.009, i.e. by 95.2%.
Keywords: bacterial consortium; microbial antagonism; *Bacillus subtilis*; bacterial preparations

Earlier monocomponent products containing one strain of a microorganisms were widely distributed on the market of microbial preparations (probiotics, plant protection products, disinfectants, etc.) In recent years, multicomponent preparations containing several symbiotic strains of prokaryotes of the same or different species are gaining increasing popularity due to their heightened efficiency. Bacteria that are included in a multicomponent preparations have a synergistic action and exchange metabolic products between each other (vitamins, amino acids, etc.), that's why the biotechnological properties of the whole microbial composition are significantly increased. In addition, such bacterial complexes have enhanced resistance to the effects of external factors like the influence of opportunistic pathogens (Artyukhova et al., 2014).

Some scientists have experimentally proved the advantages of multi strain microbial preparations over preparations containing only one strain of microorganisms. Thakkara and Saraf (2015), in their experiments to study the antagonistic activity of a bacterial consortium consisting of *Pseudomonas aeruginosa*, *Bacillus cereus*, *B. amyloliquefaciens* and *Trichoderma citrinoviride*, concluded that plants which were treated with a consortium + pathogen showed a lower incidence of disease than the control group of plants which was treated with one type of antagonist microorganism + pathogen.

Maiyappan and et al. (2010) found that the consortium "Omega" developed by them, consisting of 9 different strains of microorganisms (*Bacillus* spp., *Streptomyces* spp., *Azotobaacter* spp. и *Frauteria* spp.), was not only antagonistically active against fungal phytopathogens, but also contributed to a more significant increase in the length of the shoot compared with the case of use of chemical fertilizer.

Maxim E.A. (2014) concluded that the combined use of probiotics «Bacell», «Monosporin» и «Prolam» can significantly improve the fish productivity of juvenile carp compared to using only one of these preparations. This effect was achieved due to the fact that fishes obtained a multi-component consortium of antagonistically active microorganisms (*B. subtilis, Lactobacillus* spp., *Lactococcus* spp., *Bifidobacterium* spp., *Ruminococcus albus*) contained in various probiotic preparations.

After a series of experiments Ziaei-Nejad et al. (2006) concluded that the use of a spore probiotic containing 5 strains of bacteria from the genus *Bacillus* (*B. subtilis, B. licheniformis, B. polymyxa, B. laterosporus* и *B. circulans*) promoted increase the survival rate of shrimp in comparison with the control, as well as more efficient absorption of food due to the production of various enzymes by microorganisms.

As can be seen from the above examples, bacteria of the genus *Bacillus* are one of the most frequently used component of microbial consortia, both in crop production and animal husbandry, as well as in aquaculture. These microorganisms are characterized by high biological activity, a wide range of antagonistic action (against insects, bacteria, fungi, viruses) and, as a result, high competitiveness, as well as viability due to the formation of endospores (Baruzzi et al., 2011; Lazovskaya et al., 2013; Sorokulova, 2013; Bernardeau et al., 2017). Some species of bacilli (*B. subtilis* (Burruano et al., 2009; Sverchkova & Kolomiets, 2014), *B. licheniformis* (Dischinger et al., 2009; Nigris et al., 2018), *B. thuringiensis* (Sanchis & Bourguet, 2008; Djenane et al., 2017), etc.) are studied and used in agriculture for a long time. However, in recent years, scientists are trying to expand the pool of species of the genus *Bacillus*, which can be used in agriculture. Now the safety and antimicrobial activity of

such species as *B. pumilus* (Agarwal et al., 2017; Truong et al., 2017), *B. toyonensis* (Kantas et al., 2015; Roos et al., 2018), etc. are actively studied.

The relevance of this study for the agro-industrial complex and other sectors of the national economy, in view of all these facts and the need for regular rotation of strains in the composition of biological products to maintain their effectiveness, is no doub.

The purpose of this research was to study the antimicrobial action of a bacterial consortium consisting of strains of the genus *Bacillus*.

Materials and methods

Strains of *Bacillus* spp

The microorganisms used in this study are listed in Table 1.

Table 1. The strains of *Bacillus* spp used in the research

No of strain	Strain of microorganisms	Source
1	*B. subtilis* B-1323	VKPM*
2	*B. subtilis* B-2895	VKPM*
3	*Bacillus sp.* H3	rhizosphere of the g. *Helianthus*
4	*Bacillus sp.* C4	rhizosphere of the g. *Cichorium*

* Russian National Collection of Industrial Microorganisms

Identification of rhizobacterial strains (H3 and C4) up to the genus *Bacillus* was carried out in accordance with the Bergey's Manual (Vos et al., 2009). The safety of the isolated strains was established by the reaction to lecithinase. It is negative for non-pathogenic representatives of the genus *Bacillus*.

Test-culture

Antagonistic activity was tested against the strain of *Escherichia coli* from collection of microorganisms of Engineering Center «Prombiotech». This bacterium was isolated from the waste products of chickens from the poultry farm.

Nutrient media and culture conditions

L-broth was used for the accumulation of biomass of bacteria of the genus *Bacillus* and *E. coli*, as well as conducting experiments on the study of direct antagonism. L–broth is composed of yeast extract (5 g/L), peptone (15 g/L), NaCl (5 g/L). Solid L–medium obtained by adding agar (15 g /L) to L–broth was used to assess the purity of bacterial cultures and to study delayed antagonism. To determine the number of *E. coli* was used prepared Levine's medium.

Cultures of *Bacillus* spp. in L-broth were grown at 37°C on shaker-incubator «Innova 44» (250 rpm) for 18-24 h. Culture of *E. coli* in liquid and agarized media, as well as plates to study delayed antagonism, were grown on a thermostat «Binder BD 115» at 37 °C for 18–24 hours. Flasks for the study of direct antagonism were cultivated at 25 ± 1 ° C for 21 days (Irkitova et al., 2018).

Obtaining of microbial consortium

Previously, in different flasks, the biomass of each strain of the genus *Bacillus* was grown. On the day of the experiment, all strains of g. *Bacillus* were mixed in a sterile tube in the ratio 1:1:1:1. The obtained multicomponent microbial suspension was used in experiments to study antagonism.

Antimicrobial study

Perpendicular bands method. A multicomponent microbial suspension was inoculated as a line on the surface of a nutrient media and incubated at 37 °C for 24 h. Thereafter test-culture was inoculated as a perpendicular line to the *Bacillus* culture. The plates were incubated for 24 h at 37 °C. The antagonistic activity was detected as a zone of pathogen' growth inhibition.

Agar wells method. Pour plate method was used for inoculation of *E. coli* culture in L–medium. Plates were allowed to solidify at room temperature. Then 5-7 mm diameter wells were made in each plate with the help of a drill and 30–40 µl of multicomponent microbial suspension were charged in the wells. The plates were placed in a refrigerator for diffusion of inhibitory compounds from the well into the thickness of the agar for 24 hours. Next, the plates were incubated on a thermostat at 37 ° C for 24 hours. The radius of inhibition zone around the charged wells was recorded after incubation.

Agar blocks method. Spread plate method was used for inoculation of *Bacillus* spp. consortium in L–medium. Then plates were incubated on a thermostat at 37 °C for 24 hours. Next, the agar blocks with the grown bacillus culture were cut out with a help of a sterile drill and placed on the surface of the agar medium with *E. coli* culture inoculated by pour plate method. After this, the same actions starting at the fridge were repeated as in the case of the agar wells method. The radius of inhibition zone around the agar blocks was recorded after incubation (Irkitova & Kagan, 2012).

Testing in liquid nutrient media (method of direct antagonism). A multicomponent suspension from *Bacillus* spp. strains and test-culture were mixed in a ratio 1:1 in 100 ml of sterile L–broth. Only the culture of *E. coli* was added to the control flask in the same volume as in the experimental flask. The number of colony-forming units (CFU) of *E. coli* were checked for 1, 2, 7, 14 and 21st days in the experimental and control flasks. For this, ml^{-1} of nutrient broth with bacteria was inoculated by spread plate method in Levin's medium and incubated at 37 °C for 24 hours. Counting colonies were made in plates with 7-9 dilutions of the bacteria culture.

Analysis of results was carried out by the growth blocking index (GBI) which was determined by the following formula:

$$GBI = \frac{CFU_1}{CFU_2}$$

where CFU_1 – the number of CFU of *E. coli* in the experimental flask, CFU_2 – the number of CFU of *E. coli* in the control flask. About the presence of the antagonistic effect can be said at GBI <1 (Lazovskaya et al., 2010).

The arithmetic average of the zones of growth inhibition of the test culture (M), the standard deviation (m) and the coefficient of variation (v) were determined for the methods of delayed antagonism. The sampling was considered homogeneous at v ≤ 33%.

Results

Identification of rhizobacterial strains of *Bacillus* spp

The microscopic study of microorganisms from rhizosphere showed these bacteria to be Gram-positive rods located singly, in pairs, less often in chains. Cells of strain H3 were about 2 μm in diameter. Rods of strain C4 were less than 1 μm in diameter. The both strains produced oval endospores (Fig. 1).

The colonies of strain H3 in L–medium were dirty-white, shiny, raised, undulate and about 1.0–1.2 cm in diameter (Fig. 2, A). The colonies of strain C4 in L – medium were white (edge more transparent), circular, raised, undulate and about 0.5–0.8 cm in diameter (Fig. 2, B).

Besides of this the both strains were aerobically, catalase-positive, lecithinase-negative and also negative by Gregersen reaction. These data indicated that tested strains belong to *Bacillus* genus.

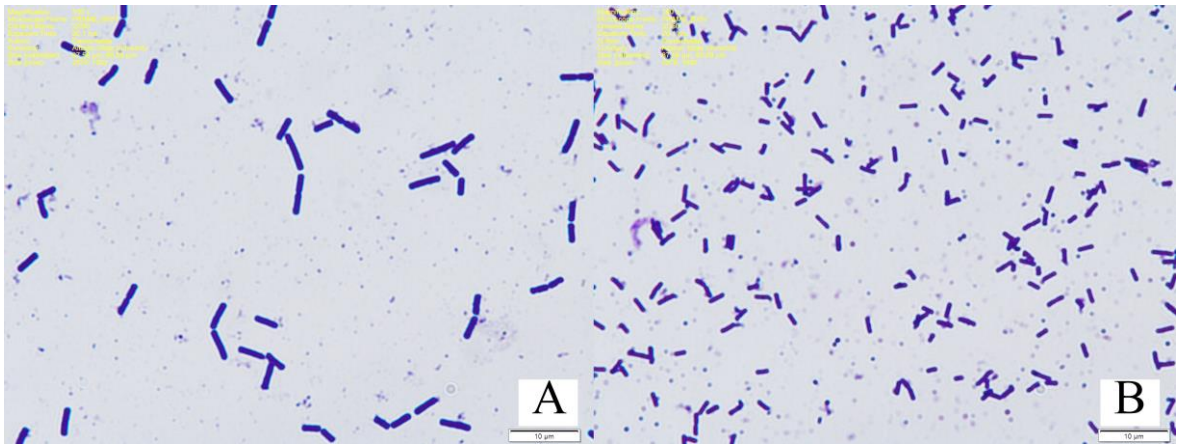

Figure 1. Gram- positive cells and spores of natural strains of *Bacillus* sp. A. Strain H3. B. Strain C4.

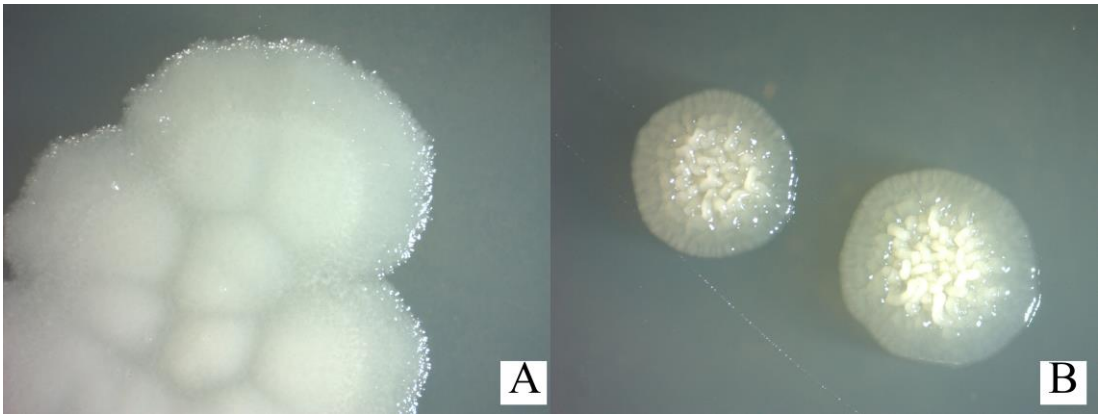

Figure 2. The morphology of the colonies of natural strains of *Bacillus* sp. A. Strain H3. B. Strain C4.

Antimicrobial action of the consortium of bacilli, established by agar diffusion methods

Consortium containing strains of the genus *Bacillus* inhibited the growth of *E. coli* in all techniques for researching delayed antagonism. The highest radius of zone of growth inhibition was recorded by the method of agar blocks (Table 2).

Table 2. Antagonistic activity of the consortium of strains of *Bacillus* spp. against *E. coli,* established by agar diffusion methods

Indicator	Agar diffusion methods		
	Perpendicular bands	Agar blocks (radius)	Agar wells (radius)
Zone of growth inhibition, mm (M±m)	2.50 ± 0.71	10.67 ±1.15	5.67 ± 0.57
The coefficient of variation *v*, %	28	11	10

The antagonistic effect of the microbial composition against test-culture was improved in compared to the single action of each strains included in the consortium. The antagonistic activity of B-1323, H3 and C4 strains of g. *Bacillus* separately, recorded by the method of perpendicular bands, was absent (and positive by the method of agar wells and agar blocks). And only for the *B. subtilis* B-2895 strain, a zone of inhibition of *E. coli* growth (2.00 mm) was observed according by method of perpendicular bands The consortium containing all 4 strains of bacilli not only retained the antagonistic effect of the *B. subtilis* B-2895 strain, but also improved it by 25%. Antimicrobial action of the consortium established by the agar block method was improved by 60.5% compared with the average value for this indicator for each of the bacilli strains separately (earlier the inhibition zone radius was 6.65 mm). And only antagonistic activity of the consortium established by the agar wells method was degraded by 10.4% compared with the average value for this indicator for each of the bacilli strains separately (earlier the inhibition zone radius was 6.33 mm).

Antimicrobial action of the consortium of bacilli, established by testing in liquid nutrient media

The microbial composition inhibited the growth of *E. coli* stably over the entire duration of the experiment in the test-flask. Although the value of GBI varied, it was always less than 1 (Table 3).

Table 3. Antimicrobial action of the bacterial consortium against *E. coli*, established by the method of direct antagonism

Day	CFU/ml^{-1} in the control flask	CFU/ml^{-1} in the experimental flask	GBI
1	51×10^{8}	13×10^{8}	0.255
2	122×10^{8}	96×10^{8}	0.787
7	249×10^{9}	128×10^{9}	0.514
14	64×10^{9}	6×10^{8}	0.009
21	25×10^{10}	45×10^{9}	0.180

As shown in Figure 3, the best antimicrobial effect of the bacterial consortium (the lowest value of GBI) was recorded on the 14th day, and the worst (the highest value of GBI) - on the 2nd day.

It should also be noted that the number of CFU in the experiment compared with the control differs by an order of magnitude (Fig. 4).

When testing the antimicrobial action of the bacterial consortium in a liquid medium, an improvement in the inhibitory effect on pathogen is noted compared to the average result for this method of studying antagonism for each of the *Bacillus* spp strains separately (GBI after 2 days was 2.645; after 14 days was 0.188; after 21 days was 0.228). So, by the 2nd day of the experiment, the antimicrobial activity of the consortium contributed to the increased blocking of *E. coli* growth by 70.2%, and by the 14th day - by 95.2%. On the third week the most pronounced antagonistic effect of the microbial composition in comparison with individual strains was also shown but only by 21%.

Discussion

In the present study, two bacterial strains isolated from rhizosphere of plants on the basis of microscopy and morphology, and biochemical and physiological tests were identified as *Bacillus* sp. H3 and *Bacillus* sp. C4.

In general, the antibacterial effect of a consortium of *Bacillus* spp strains against *E. coli* using delayed antagonism methods, was improved in comparison with the average value of the results of each of the bacilli strains separately. This means that in the consortium of collectible and natural strains of the genus *Bacillus*, the bacteria mutually reinforce and complement each other's biological activity, i.e., synergy is observed (Tsigarida et al., 2003).

A slight decrease in the antagonistic effect for the consortium in the agar wells method is most likely due to the fact that each method of studying the antibacterial action of microorganisms triggers different mechanisms of microbial antagonism, which are primarily associated with the synthesis of antibiotics and other metabolic products (Feichtmayer et al., 2017). Antimicrobial compounds can be low-molecular and high-molecular (Baruzzi et al., 201), that directly affects the possibility and rate of their diffusion in liquid and solid nutrient media. The agar well method is fundamentally different from the methods of studying antagonistic activity using agar blocks and perpendicular bands. This is due to the fact that only in this case the bacterial consortium being in a L–broth is incubated in the Petri dish inside the wells. In a liquid nutrient medium, high molecular weight organic compounds can accumulate, but not the fact that they can diffuse into the layer of agar in which the well was drilled (Valgan et al., 2007).

All this indicates that in order to obtain the most complete and accurate data on the level of antagonistic activity of a bacterial consortium or a concrete strain, it is necessary to apply various methods of studying microbial antagonism.

Testing the antibacterial action of the *Bacillus* spp consortium by direct antagonism under conditions different from optimal for bacilli (deficiency of oxygen) and *E. coli* (low temperature) allows for increased competition between bacteria for the nutritional substrate. As a result, different strains of the genus *Bacillus* begin to actively produce antibiotic and other compounds that inhibit the growth of the pathogen (Hibbing et al., 2010).

The worst GBI value on the 2nd day of the experiment indicates that at this stage, inhibitory substances produced by bacilli have not yet accumulated in a liquid nutrient medium. And the best growth blocking index on the 14th day of the experiment indicates the duration of the effect of inhibition of pathogen. That must be considered when developing bacterial preparations. The increase of GBI in the third week means that the *Bacillus* spp. strains due to the lack of nutrients and oxygen began sporulation (Jabbari et al., 2011). That led to a decrease in the antagonistic effect.

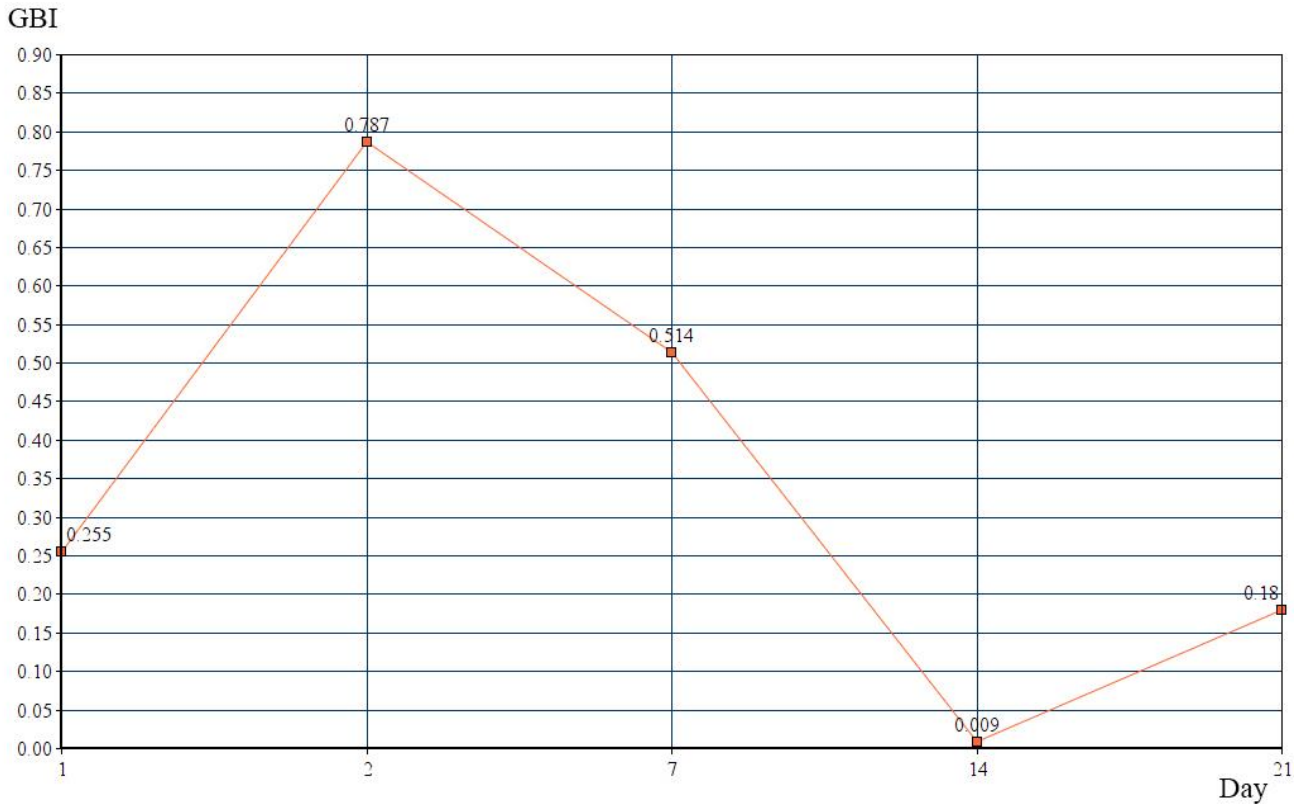

Figure 3. The change in the value of GBI during the experiment

Based on the conducted research, it can be argued that the microbial association has a more pronounced antagonistic effect than a single bacterium. In this regard, the bacterial consortium developed by us, consisting of collectible and natural strains of *Bacillus* spp, after further study of its safety can be recommended for inclusion in the composition of biological preparations.

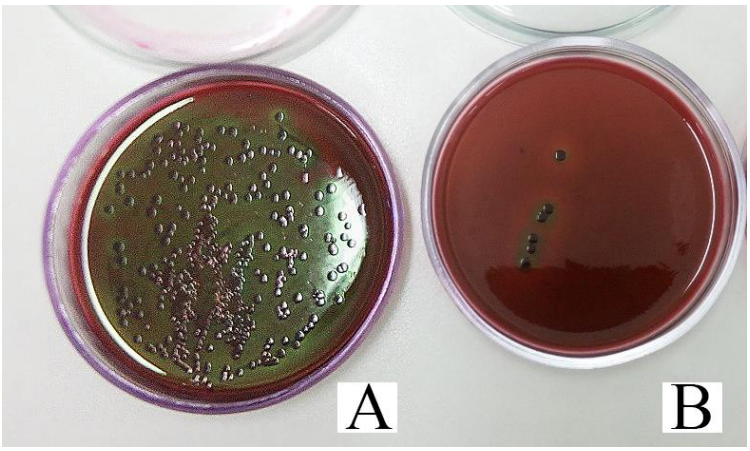

Figure 4. The number of *E. coli* on the 14th day of test. A. In the control flask. B. In the experimental flask

Conclusion

The antimicrobial effect of the developed bacterial consortium from *Bacillus* spp strains was recorded by all the methods used in the study. Besides the antagonistic effect of the microbial composition against *E. coli* was improved in compared to the single action of each strains included in the consortium. In agar media, the largest increase of the radius of zone of growth inhibition was noted from 6.65 mm to 10.67 mm, i.e. by 60.5%, and in liquid media – an improvement in the growth blocking index from 0.188 to 0.009, i.e. by 95.2%. Therefore, the method of direct antagonism allows better assess the potential of antimicrobial action of this consortium.

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