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The Effects of Commercial Probiotics on Biological Properties of Enterococci in Colon Carcinoma Cell Culture

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Abstract

Background: The interactions between dysbiosis of gut microbiota and the development of colorectal cancers are well known. We investigated the effects of three different commercial probiotics, purchased from pharmacies, on several biological properties of enterococci (vancomycin-resistant "VRE" and vancomycin-susceptible "VSE") in colon adenocarcinoma cell culture (HT-29).

Methods: Cell-free supernatants (CFSs) were prepared after isolating probiotic strains (*Bifidobacterium lactis*, *Saccharomyces boulardii,* and *Bacillus clausii*). Bacterial growth was detected spectrophotometrically after three, six, and 24 hours of incubation. Adhesion and invasion assays were performed using the colony counting method. Biofilm formation was performed using microtiter plate assay. GraphPad Prism software, version 5, was used for analysis.

Results: After 24 hours incubation in culture medium, all three probiotics increased (P<0.001) the growth of VRE and VSE. Bacterial growth was also increased in cell culture in the presence of probiotics (P<0.001). Adhesion of both enterococci was reduced (P<0.001) by all probiotics. The invasion and biofilm formation were varied according to strains and probiotics tested.

Conclusion: The effects of probiotics may vary depending on the specific strains used. Furthermore, during probiotic usage, potential risk of enhanced pathogenicity under certain circumstances, especially in immune suppression should be considered.

Keywords: Colorectal cancer, Probiotics, Enterococcus, Biofilms

Introduction

The crosstalk between gut microbiota and host plays a crucial role in maintaining the homeostasis by modulating the immune system, producing organic acids, bacteriocins, free oxygen radicals, antimicrobial peptides, short chain fatty acids, secondary bile acids, and competing with pathogens via binding receptors. Nearly 80-90% of gut microbiota are classified in *Bacteroides* and *Firmicutes* phyla. The composition of gut microbiota is crucial for maintaining the host's health. However, alterations of this composition, which is called dysbiosis, could lead to various pathologies such as

metabolic and inflammatory diseases and different types of cancers (1-10).

Microbial dysbiosis stimulates the pathological transformations in colon cells by modulating cell proliferation, apoptosis and immune responses which emphasize the close relationship between cancers and microbes. Some gut microbiota members such as, *Streptococcus gallolyticus* (formerly *S. bovis* biotype 1), *Clostridia* and *Bacteroides fragilis* are proven to have crucial role on cancer promotion. However, some others such as *Escherichia coli* and *Enterococcus faecalis* could play a role depending on predisposing factors and their rational balance (1, 2, 11-14). These microbes can generate various metabolites (bile acids, short-chain fatty acids, indole and amino acid metabolites etc.) which are associated with development of colorectal cancer (CRC) and also intestinal polyps (1, 2, 4, 7, 11, 15-17).

As a member of gut microbiota, enterococci can cause life threating infections (blood stream infections, periodontitis, endodontic diseases, urinary tract infections and endocarditis) especially in immunosuppressed and hospitalized patients (9, 18-21). On the other hand, *E. faecalis* defined as a "driver bacteria" can lead to the development of CRC and adenomatous polyps by producing free oxygen radicals such as superoxide, hydrogen peroxide and hydroxyl radicals which are highly mutagenic and causes chromosomal instabilities by damaging host cell DNA (2, 4-6, 9-11, 16, 17). Consistently, one study showed that Biliverdin, a metabolite of *E. faecalis* promotes the growth of CRC cells and also stimulates angiogenesis (11).

Considering these effects, we aimed to investigate the interactions between Enterococci, one vancomycin-susceptible VSE and one vancomycin-resistant VRE, and colon adenocarcinoma cells (HT-29) in the presence of three different commercial probiotics.

Materials and Methods

All experiments (isolation of the probiotic strains, growth alterations, adhesion, invasion, and biofilm formation assays) were performed in İstanbul Yeni Yüzyıl University's laboratories between December 2023 and February 2024.

Strains, Different Commercial Probiotics and Cell Culture

Two different Enterococci (one vancomycin susceptible and one resistant), isolated in January 2024 from two different patients hospitalized in Istanbul University, Medical Faculty, Autologous Stem Cell Clinic, were examined. Enterococci (VSE and VRE) were grown in Tryptic Soy broth (TSB) at 37°C for 24 hours. Initial concentrations of bacteria were prepared as 10^7 CFU/mL for all experiments.

Probiotic microorganisms that contain only one type of probiotic microorganism were chosen randomly and purchased from pharmacies. Probiotic microorganisms were defined as *Bifidobacterium lactis* (CFS-1), *Saccharomyces boulardii* (CFS-2) and *Bacillus clausii* (CFS-3) by manufacturer and in the present study, the Cell-free supernatants (CFSs) were obtained.

For isolation of probiotic strains, brain heart infusion broth was used and cultures were incubated at 37°C for 24 hours. The overnight cultures of probiotics were centrifuged at 5000 rpm for 10 minutes and they were filtered with 0.2 µm pore size filters to prepare CFSs which were used in all experiments (22, 23).

Human colon adenocarcinoma cells (HT-29) (91072201-1VL, Sigma-Aldrich, USA) were used in our experiments and Dulbecco's modified Eagle medium (DMEM) (Sigma, 5546, USA) supplemented with 10% fetal bovine serum (FBS) (Biowest, S1810-500, France), 1% 2mM L-glutamine (Biological Industries, BI03-020-1B, Israel) and 1% penicillin/streptomycin (50 IU/ml penicillin and 50µg/ml streptomycin; Biological Industries, 03-031-1B, Israel) was used for cell culture. Colon cells were seeded in 96-well microplates for bacterial growth, seeded in 24-well plates to detect adhesive and invasive properties of Enterococci. To provide a confluent monolayer cell culture, density was adjusted as approximately 5×10^4 cells for 24-well and 1×10^4 cells for 96-well plates. The plates were incubated at 37°C, under 5% CO_2 conditions for 24 hours (22, 23).

Inoculation of Cell Culture with Two Different Enterococci

Initial concentrations of Enterococci cultures were adjusted as 10^7 CFU/mL for all experiments. Before inoculating Enterococci, a medium supplemented with antibiotics was removed from the cell culture, and antimicrobial solution-free DMEM was added to each well. Enterococci were added (20µL for each well/96-well plates and 50 µL for each well/24-well plates) into cell culture (22, 23).

The Effects of CFSs on the Growth of Enterococci in the Presence of HT-29 Cell Culture

Infected cells were incubated in the presence/absence of each CFS for 24 hours at 37°C for detecting alterations of growth. Bacterial growth was determined by measuring the changes in absorbance at 600 nm after three, six, and 24 hours of incubation. The absorbance results of infected HT-29 cell cultures with/without CFSs were compared. The experiments were repeated three times independently and all conditions were analyzed three times (22, 23).

The Effects of CFSs on Adhesion and Invasion of Enterococci

Enterococci inoculated cells were incubated in the presence/absence of CFSs at 37°C for three hours. Following the adhesion and invasion assay stages

(mentioned below), the effects of each CFS were detected by comparing colony counts (as CFU/mL) obtained from cell lysates of cell cultures. Colonies of adherent or invasive bacteria were counted from Tryptic soy agar after 24 hours of incubation at 37°C. All conditions were performed at least three biological replicates and three technical replicates were also considered (22-25).

Bacterial Adhesion

After incubation for three hours as mentioned above, inoculated HT-29 cells were washed three times with phosphate buffer saline (PBS) to remove unbound bacteria and treated with 500µl Triton X-100 (0.025%) to lyse the cells for 10 minutes at 37°C. The real number of adherent bacteria was detected by colony counting method. We homogenized the cell lysates and inoculated on Tryptic soy agar at 37°C under aerobic conditions (22-25).

Bacterial Invasion

Following incubation at three hours as mentioned above, infected HT-29 cells in wells were washed with PBS three times and a fresh medium containing 200 µg/mL gentamycin was added to kill extracellular bacteria, and plates were incubated at 37°C for one hour. Then, HT-29 cells were lysed with Triton X-100, and for quantification of invasive bacteria; cell lysates were homogenized and inoculated as performed in the adhesion assay (22-25).

The Effects of CFSs on Biofilm Formation of Enterococci

Microtiter plate assay was used to detect biofilm formation. Overnight cultures of VRE and VSE were cultured in glucose (1% v/v) and TSB was added at 37°C. Before the experiments, cultures were diluted to prepare a final concentration of 10^7 CFU/mL.

100µL of bacterial suspension and 100µL of each CFS were added into the wells. *E. coli* ATCC 25922 (a biofilm-forming strain) was used as positive control and un-inoculated TSB-glucose was used as negative control (22, 26).

After incubation of a 96-well cell culture microtiter plate for 24 hours at 37°C wells, waste media were gently washed three times with sterile phosphate-buffered saline (PBS) solution. For fixation, 200µL of methanol (99%) was added to air-dried wells. After fixation with methanol for 15 minutes, it was

aspirated and the plates were allowed to dry. Wells were stained with 200µL 0.1% crystal violet (diluted in distilled water) for 5 minutes at room temperature. Excess stain was gently washed with tap water and the plates were air dried. By adding 200µL ethanol (95%) the stain was re-solubilized. After incubating the template for 10 minutes at room temperature the optical density was measured at 450 nm. The results were interpreted comparing with positive and negative control. All experimental conditions were analyzed four times (22, 26).

Statistical Analysis

The effects of CFSs, HT-29 cells, and their co-presence on Enterococci were statistically analyzed on GraphPad Prism software 5 (Dotmatics, USA) by comparing control conditions to experimental conditions. Growth alterations were carried out using two-way ANOVA followed by Bonferroni posttests. Besides, one-way ANOVA followed by Tukey's multiple comparison tests was used for the analysis of adhesion, invasion, and biofilm properties. All results were presented as mean ± SD. Differences with p-values less than 0.05 were considered significant.

Results

The Effects of CFSs on the Growth of Enterococci

According to the results, CFS-2 and CFS-3 significantly decreased the growth of VSE (P<0.001 and P<0.01, respectively) during six-hour incubation. On the other hand, when the incubation time increased to 24 hours each three CFSs induced the growth of VSE (P<0.001, Figure 1a).

CFS-1 significantly induced (P<0.001) the growth of VRE both at six and 24 hours of incubation. CFS-3 also induced the growth of VRE (P<0.001) only at 24 hours. On the contrary, CFS-2 decreased the growth of VRE in both six (P<0.01) and 24 hours (P<0.05, Figure 1b).

The Effects of CFSs on Growth of Enterococci in the Presence of HT-29 Cell Culture

The CFS-3 was shown to significantly induce (P<0.001) the growth of VSE at the six-hour incubation in the presence of HT-29 cell culture. Besides, when the incubation was prolonged, both CFS-1 and CFS-3 induced the growth of VSE (Figure 2a).
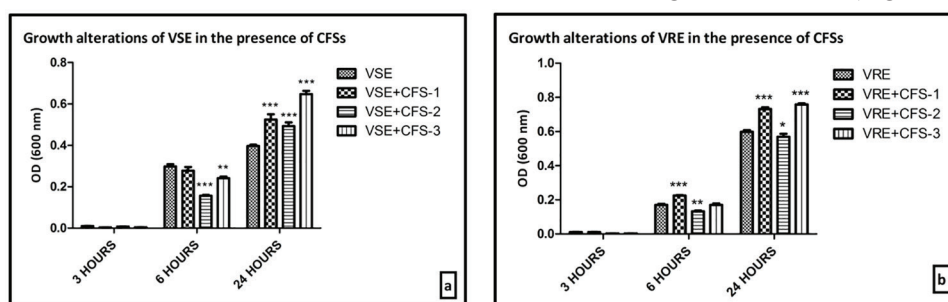

Figure 1: Growth alterations of VSE (a) and VRE (b) in the presence of CFSs. VSE: Vancomycin susceptible; VRE: Vancomycin resistant; CFS: Cell-free supernatant; *P<0.05; **P<0.01; ***P<0.001
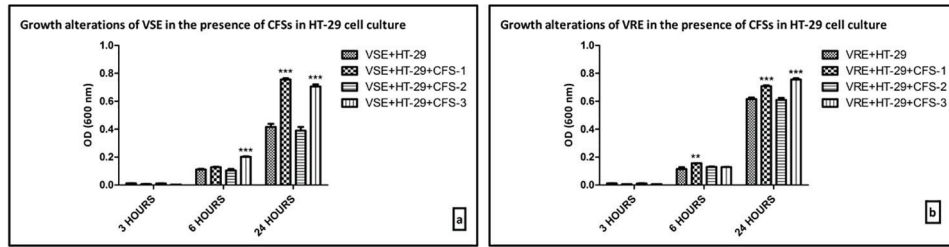
Figure 2: Growth alterations of VSE (a) and VRE (b) in the presence of three CFSs in HT-29 cell culture. VSE: Vancomycin susceptible; VRE: Vancomycin resistant; CFS: Cell-free supernatant; HT-29: Human colon adenocarcinoma cell line; **P<0.01; ***P<0.001
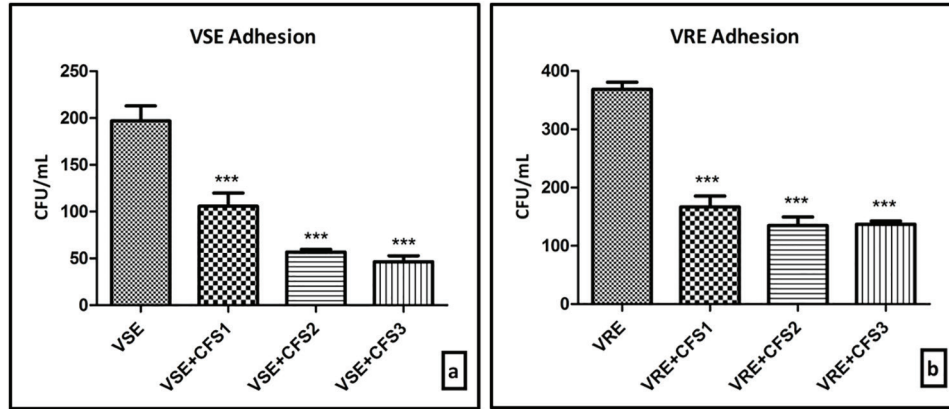

Figure 3: Alterations on adhesion of VSE (a) and VRE (b) in the presence of three CFSs. VSE: Vancomycin susceptible; VRE: Vancomycin resistant; CFS: Cell-free supernatant; ***P<0.001
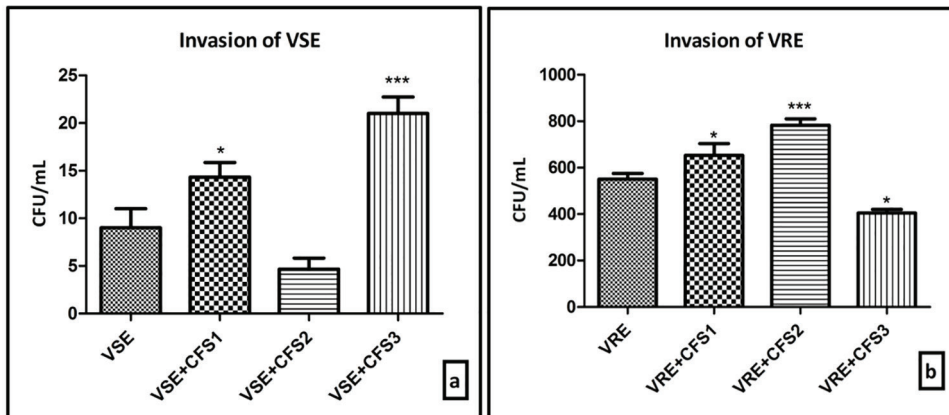

Figure 4: Alterations on invasion of VSE (a) and VRE (b) in the presence of three CFSs. VSE: Vancomycin susceptible; VRE: Vancomycin resistant; CFS: Cell-free supernatant; *P<0.05; ***P<0.001

The growth of VRE significantly increased (P<0.01) in the presence of CFS-1 and HT-29 cells at six hours. Besides, both CFS-1 and CFS-3 significantly increased (P<0.001) the growth of VRE in cell culture conditions at the 24-hour incubation (Figure 2b).

Furthermore, it was investigated that the growth of both VRE and VSE were significantly reduced (P<0.01 and P<0.001, respectively) in the presence of HT-29 cells without CFSs at six-hour incubation.

The Effects of CFSs on Adhesion and Invasion of Enterococci

It was shown that, each three CFSs were significantly reduced the number of both adherent VSE and VRE (P<0.001) (Figure 3a and 3b).

CFS-1 and CFS-3 significantly induced invasion properties of VSE (P<0.05, P<0.001, respectively) (Figure 4a). Although the number of invasive VRE

significantly increased in the presence of CFS-1 (P<0.05) and CFS-2 (P< 0.001), CFS-3 decreased the invasion of VRE (P<0.05, Figure 4b).

The Effects of CFSs on Biofilm Formation of Enterococci

Biofilm formation of VSE was significantly suppressed in the presence of CFS-1 (P<0.01) and CFS-3 (P<0.05) (Figure 5a). Reversely, the biofilm formation of VRE was induced in the presence of CFS-2 (P<0.05) and CFS-3 (P<0.001, Figure 5b).

Discussion

Probiotics, defined as "live microorganisms administrated in adequate amounts in food or as dietary supplement which promote positive physiological effects on the host", can support the host's resistance mechanisms against intestinal
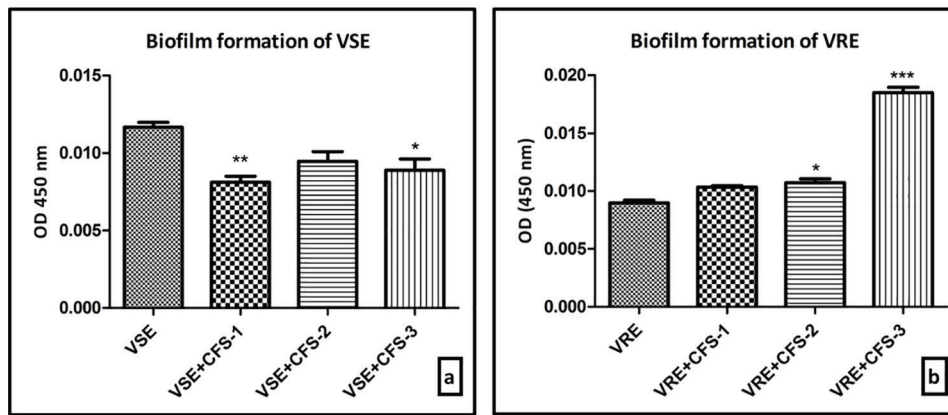
Figure 5: Alterations on biofilm formation of VSE (a) and VRE (b) in the presence of three CFSs. VSE: Vancomycin susceptible; VRE: Vancomycin resistant; CFS: Cell-free supernatant; *P<0.05; **P<0.01; ***P<0.001

pathogens. According to this phenomenon, a probiotic organism, as a member of microbiota, should be specified as "Generally Recognized as Safe" status which *Lactobacillus*, *Bifidobacteria,* and *Bacillus* are the most common ones. As a current therapeutic approach, their usage has become useful to overcome various infectious diseases such as infantile diarrhea, necrotizing enterocolitis, antibiotic-associated diarrhea, *Helicobacter* infections, oral health diseases, and traveler's diarrhea. (19, 20, 27-30).

Enterococci reside commonly not only in the oral cavity and gastrointestinal tract of mammals and other animals including birds, insects, and reptiles (20, 31, 32). Some enterococci, especially VRE, could be a cause of various nosocomial infections which are also resistant to beta-lactams, glycopeptides, and aminoglycosides. Both intrinsic and acquired resistance lead to the expansion and persistence of these resistant strains into the gut which is associated with an increased risk of invasive infections (5, 9, 18, 20, 21).

In this light of information, many studies have focused on the relations between probiotics (mostly lactobacilli and/or their products) and enterococci as an opportunist pathogen. Although there are a limited number of studies reporting that probiotics have no beneficial effects on removing and/ or inhibiting enterococci (19, 30). Studies show probiotics decrease the growth of enterococci and could be considered as a potential therapeutic agent against enterococci as mentioned below (REF).

L. rhamnosus GG strain prevents the colonization and overgrowth of VRE. Even though the *in-vivo* anti-VRE effect of *L. paracasei* CNCM I-3689 strain was shown, via lithocholate and propionate secretion, *in-vitro* conditions did not support this interaction. On the other hand, *L. paracasei* CNCM I-3689 was shown to support the presence of *Bacteroidetes*, also a propionate producer, when antibiotic-induced dysbiosis occurred (21).

In terms of treatment of oral infections, the secreted products of *L. casei* and *L. plantarum* prevented the regrowth of *E. faecalis's* biofilms. Therefore, researchers have suggested that lactobacilli or their products were efficient and safe therapeutic agents to eliminate *E. faecalis* from the root canal to treat apical periodontitis (20).

In another study *L. rhamnosus*, *L. acidophilus*, *L. casei* inhibited the growth of both *E. faecalis* and *C. albicans in-vitro* which also supported the potential role of probiotics in treatment of endodontic infections (33). Kalaycı-Yüksek et al. have shown that the CFSs of *L. fermentum*, *L. acidophilus, L rhamnosus* and *L. plantarum* inhibited the growth of 30 different VRE strains (34).

It is well known that colonization is one of the crucial steps of pathogenesis for all bacteria. The most important virulence mechanism of enterococci is also defined as colonization capacity. Because ~99% of homology between pili of VRE (PilB) and lactobacilli (SpaC) was shown; it was reported that the strong binding capacity of *L. rhamnosus* GG to mucus effectively displaces *E. faecium* from mucus (18).

As one of the rare examples investigating probiotics other than lactobacilli, *B. clausi*'s CFS was shown to have inhibitory effects on *E. faecalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter baumanii* and their antitoxic activities were also shown against *Clostridium difficile* and *Bacillus cereus* (35, 36). In addition, *Bacillus coagulans* was found to be ineffective in regrowth of *E. faecalis*'s biofilms (20).

The presence or absence of a unique organism as well as specific relations between various strains is essential for the development of CRC. Thus, fecal compositions were compared in CRC patients and healthy individuals in several studies (1, 2, 7, 11, 12). In one of these studies done by Elahi et al. (2023) it was shown that, in tumor tissues, the number of *Bifidobacterium*, *Lactobacillus*, *Clostridium* and *Firmicutes* was significantly reduced, whereas the number of *Enterococcus* was shown to be increased. The authors also emphasized that *Firmicutes*, butyrate producers, are crucial to prevent CRC via increasing colon regulatory T cells and decreasing inflammatory cytokines (7). In line with previous studies (1, 2, 7, 11, 12), Geravand and colleagues showed that the average number of *E. faecalis* in patients with CRC was higher than the number in patients with polyps

and healthy individuals (2). On the other hand in another study, fecal samples of CRC patients were demonstrated to have a reduced frequency of *E. faecalis* compared to healthy individuals (77% versus 22%). Moreover, the secreted metabolites of *E. faecalis* strains isolated from healthy individuals reduced the proliferation of three colon carcinoma derivate cell lines (HCT-8, HCT-116 and SW620) (1).

Different from many other studies, in our study, probiotics (*Bifidobacterium lactis*, *Saccharomyces boulardii* and *Bacillus clausii*) other than lactobacilli were investigated for their anti-enterococcal effects. According to growth results of 24 hours incubation, all three probiotics induced the growth of both VSE and VRE in the DMEM medium. As an exception *S. boulardii* inhibited the growth of VRE only. Furthermore, probiotics were shown to induce the growth of both VRE and VSE in HT-29 cell culture conditions except for *S. boulardii* which has no effect. It is possible to suggest that, especially the CFSs of *Bifidobacterium lactis* and *Bacillus clausii* increased the growth of enterococci, which is thought to be an important risk factor for progression of CRC.

We also found that the adhesion of bacteria was reduced while invasion and biofilm formation were altered variously. Although the CFS of *B. lactis* decreased the invasion of enterococci, the effects of other CFSs were strain-dependent. The biofilm formation of VRE was induced in the presence of *B. clausii* and *S. boulardii* but the biofilm of VSE decreased in the presence of *B. lactis* and *B. clausii.*

In this study, the fact that the effects of CFSs on enterococci were not compared with the results of another cell line might be considered a limitation.

Conclusion

In conclusion, the strong association between gut microbiota and host occurs not only via cell-to-cell interactions but also via metabolites of microorganisms and host. All of these findings indicate the potential risk of enhanced pathogenicity under certain circumstances, especially in immune suppression. Although probiotics are considered alternative therapeutic agents in the context of irrational antibiotic usage, they still should be used carefully. Furthermore, different clinical studies are needed to analyze the reliability of probiotics to avoid pathological conditions.

Acknowledgment

Conflict of interest: None declared.

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