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Chapter 10

Antimicrobial Effect of Probiotics against Common Pathogens

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Additional information is available at the end of the chapter

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Abstract

The antimicrobial or antagonistic activity of probiotics is an important property that includes the production of antimicrobial compounds, competitive exclusion of pathogens, enhancement of the intestinal barrier function and others. There are many methods to ascertain probiotic properties, including various *in vitro* and *in vivo* methods. The *in vivo* methods include various modifications of the spot-on lawn assay, agar well diffusion assay (AWDA), co-culturing methods, usage of cell lines and others. In many cases *in vitro* antagonist activity is observed, but in real settings it is not observed. The *in vivo* methods mainly used are animal models; however, their use is being restricted according to the European legislation OJ L136. The justification of animal models is also questionable as the results of studies on animals do not predict the same results for humans. The use of replacement alternative methods, for example incorporating human cells and tissues, avoids such confounding variables. Most important studies are double-blinded randomized clinical trials; however, these studies are difficult to perform as it is not easy to achieve uniform conditions. There is a clear need for more elaborate assays that would better represent the complex interactions between the probiotics and the final host. This complex situation is a challenge for scientists.

Keywords: antimicrobial effect, *in vitro* methods, *in vivo* methods, pathogens, probiotics

1. Introduction

Throughout the history of microbiology, most human studies have been focused on the disease-causing organisms found on or in people; whilst fewer studies have examined the benefits of the resident bacteria. However, we are surrounded by beneficial microorganisms that live in or

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on the human body. The intestinal microbiota is very well adapted, exceptionally stable and very specific for each individual. In normal conditions of stable functioning of the digestive system, neutral and beneficial microorganisms dominate. It is estimated that there are 100 trillion microorganisms in the intestine of a human adult and this is 10 times larger than the number of cells in the human body [1, 2]. However, the balance of the intestinal microbiota is negatively influenced by modern lifestyle, leading to increased numbers of pathogenic microorganisms that disrupt microbial balance and cause a reverse from beneficial to harmful functioning. In such cases, the external support with probiotics is very welcome and supported by several scientific studies [3].

According to the Food and Agriculture Organisation of the United Nations (FAO) and the World Health Organisation (WHO), probiotics are defined as live microorganisms, which when administered in adequate amounts confer a health benefit on the host [4, 5]. The most common probiotic bacteria are certain strains from the genera *Lactobacillus* (i.e., *L. rhamnosus, L. acidophilus, L. plantarum, L. casei, L. delbrueckii subsp. Bulgaricus,* etc.) and *Bifidobacterium* (i.e., *B. infantis, B. animalis* subsp. *lactis, B. longum,* etc.). Other probiotic bacteria include *Pediococcus acidilactici, Lactococcus lactis* subsp. *lactis, Leuconostoc mesenteroides, Bacillus subtilis, Enterococcus faecium, Streptococcus thermophilus, Escherichia coli* Nissle 1917, etc. Certain yeasts such as *Saccharomyces boulardii* are also probiotics [6, 7].

Probiotics together with other beneficial microbes are commensals of the gut and differ from pathogenic bacteria in the terms of their actions on immune cells in the gut as they do not stimulate the proliferation of mononuclear cells or trigger an inflammatory action [8]. Regardless of whether the probiotics are used for human or animal consumption, there are several characteristics that a probiotic must achieve. Some of the important characteristics of probiotics include the following: a probiotic must be generally required as safe (GRAS); a probiotic should exhibit bile and acid tolerance in order to survive the path from the oral cavity to the small intestine where it lives, multiplies and excretes beneficial nutrients and molecules; a probiotic should have the ability to adhere to mucus and/or epithelial cells, and/or other surfaces; a probiotic should be susceptible to antibiotics; a probiotic should exhibit antimicrobial activity against pathogens [3, 5, 9, 10]. Although it is accepted that probiotics must be of human origin [4, 5], many authors have found that some strains that are not normally isolated from human have shown to be effective [11, 12], which negates this requirement. As noted above, one of the important attributes of probiotics is their antimicrobial effect against pathogens by maintaining the homeostasis of the intestinal flora. Assessing the antimicrobial effect of various probiotics against pathogenic microorganisms is the guiding concept of this chapter. This chapter reviews the principles and results from various authors of different methods for determining the antimicrobial or antagonistic effect of probiotics against potential pathogens.

2. Antimicrobial or antagonistic properties of probiotics

In literature both the terms "antimicrobial" and "antagonistic" are found to determine the ability of one species to inhibit the growth of another species. According to the online Encyclopaedia Britannica, "antagonism" refers to an "association between organisms in which one

benefits at the expense of the other,." However, this encyclopaedia does not include the adverb "antimicrobial." On the other hand, it contains "antimicrobial agent" that refers to "a large variety of chemical compounds that are used to destroy microorganisms or to prevent their development.." The online Merriam Webster dictionary defines "antimicrobial" as "destroying or inhibiting the growth of microorganisms and especially pathogenic microorganisms" and "antagonistic" as "showing dislike or opposition: showing antagonism."

This antimicrobial/antagonistic ability is especially important for probiotics as one of the functional beneficial requirements of probiotics is a broad antimicrobial spectrum as well as antagonism against pathogenic bacteria with strong antimicrobial activity. The antagonistic activity of one microorganism against another can be caused by competitive exclusion, immune modulation, stimulation of host defence systems, production of organic acids or hydrogen peroxide that lower pH, production of antimicrobials such as bacteriocins, antioxidants, production of signalling molecules that trigger changes in gene expression [13–15]. Antimicrobial substances produced by beneficial microorganisms are known to include lactic acid, acetic acid, formic acid, phenyllactic acid, benzoic acid as well as other organic acids, short chain fatty acids, hydrogen peroxide, carbon dioxide, acetaldehyde, acetoin, diacetyl, bacteriocins and bacteriocins-like inhibitory substances and others [10, 16, 17]. The most common bacteriocins include lacticin, lactocin, pediocin, pisciolin, enterocin, reuterin, plantaricin, enterolysin and nisin [18, 19].

3. Methods

3.1. Methods of literature research

A literature overview of three databases was conducted using the two following keywords: "probiotic" and "antimicrobial" between the years 1980 and 2016. The search yielded 2882, 1017, and 6200 publications in PubMed, Web of Science, and Science Direct databases, respectively (**Figure 1**).

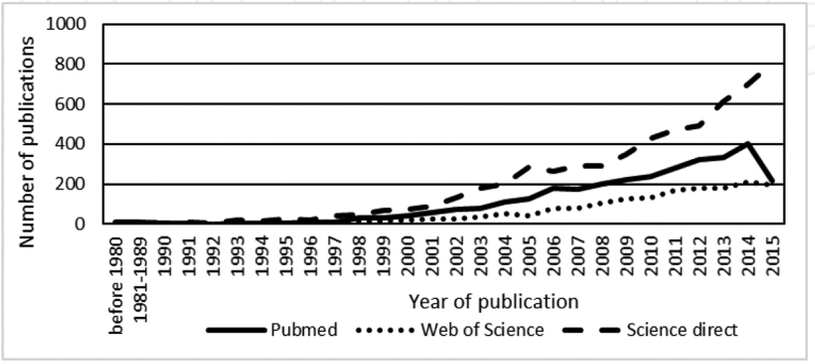


Figure 1. Results of the publications in PubMed, Web of Science and Science Direct databases using keywords: "probiotic" and "antimicrobial" between the years 1980 and 2015.

All three databases showed great increase in the number of publications in the past 10 years. The highest results were obtained via Science Direct due to the fact that this database includes various journals and books from the area of food and dairy sciences, the area of probiotics for animals as well as the area of human probiotics. PubMed on the other hand contains only research of probiotics for humans. Also, the programs for keyword searching for each chosen database seem to differ among each other thus yielding very different numbers of publications in journals, chapters and conference proceedings. The research on the methods for determining the antimicrobial effect of strain-specific probiotics was conducted by adding an additional keyword aside "probiotic." This keyword described the various investigated methods for determining the antimicrobial/antagonistic activity of probiotics (i.e., spot-on lawn, agar spot, agar well diffusion, paper disc, co-culturing, *in vivo*). Of course, it was not possible to screen every single article therefore only a selection of the most recent or relevant were used.

3.2. *In vitro* methods for determining the antimicrobial/antagonistic effect of probiotics against other microorganisms

3.2.1. Spot-on lawn antimicrobial assay/agar spot antimicrobial assay

The spot-on lawn antimicrobial assay has been described by several authors. Several modifications of the method have been made. Also various other expressions are used such as agar spot assay, critical dilution assay and deferred antagonism assay [10, 16, 20–22].

One of the simplest published principles of the spot-on lawn antimicrobial assay (**Figure 2a**) consists of the following steps: different nutrients, selective or differential media, are prepared and various chosen indicator microorganisms or pathogens at different initial concentrations are either inoculated in a confluent manner after hardening of agar or are mixed with the agar in liquid state and poured into the plate. Different dilutions of aliquots of the investigated probiotic or cell-free supernatant with bacteriocins are then spotted onto the media already inoculated with chosen indicator microorganisms [20, 23, 24].

After incubation, the antimicrobial activity is expressed either as inhibition zone or as arbitrary units (AU/mL). The zone of inhibition is noted either as the diameter or the area of the inhibition zone. The critical dilution is noted as the last dilution that produces a zone of inhibition larger than 6 mm. Arbitrary units are defined as the reciprocal of the highest dilution at which the growth of the indicator microorganism or pathogen is inhibited and are calculated as $(1000/a) \times D$ in AU/mL, where a is the aliquot of cell-free supernatant added to well in μL and D is the dilution factor [22, 24].

A modification is the agar spot antimicrobial assay (**Figure 2b**) and consists of the following steps: MRS agar or other specified agar is prepared and the probiotic bacteria or test cultures (few μL) are spotted on. These agars are then incubated to develop spots. Next, the indicator bacteria (pathogenic species, spoilage species and other probiotic species) are mixed into specific soft agar (0.7%) and poured over a plate. The plates are then incubated aerobically or anaerobically and the inhibition zones are read. A clear zone of more than 1 mm around the spot is considered as positive [25]. A third modification is the spot-on lawn antimicrobial assay with wells (**Figure 2c**), which consists of the following steps: chosen nutrients, selective or

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differential media, are prepared. Wells (6 mL, 7 mm or other dimensions) are bored in each plate and the bottom of the wells is sealed with agar. Aliquots of active cultures at different dilutions are pipetted into the wells. The plates are left at room temperature to allow migration and settling of the test cultures. The samples are then incubated for 3 h at 37°C and the plates are then overlaid with agar seeded with indicator pathogenic microorganisms (or other indicator organisms) and incubated at suitable incubation conditions. After incubation, the antimicrobial activity is expressed either as inhibition zone or as arbitrary units (AU/mL) [16].

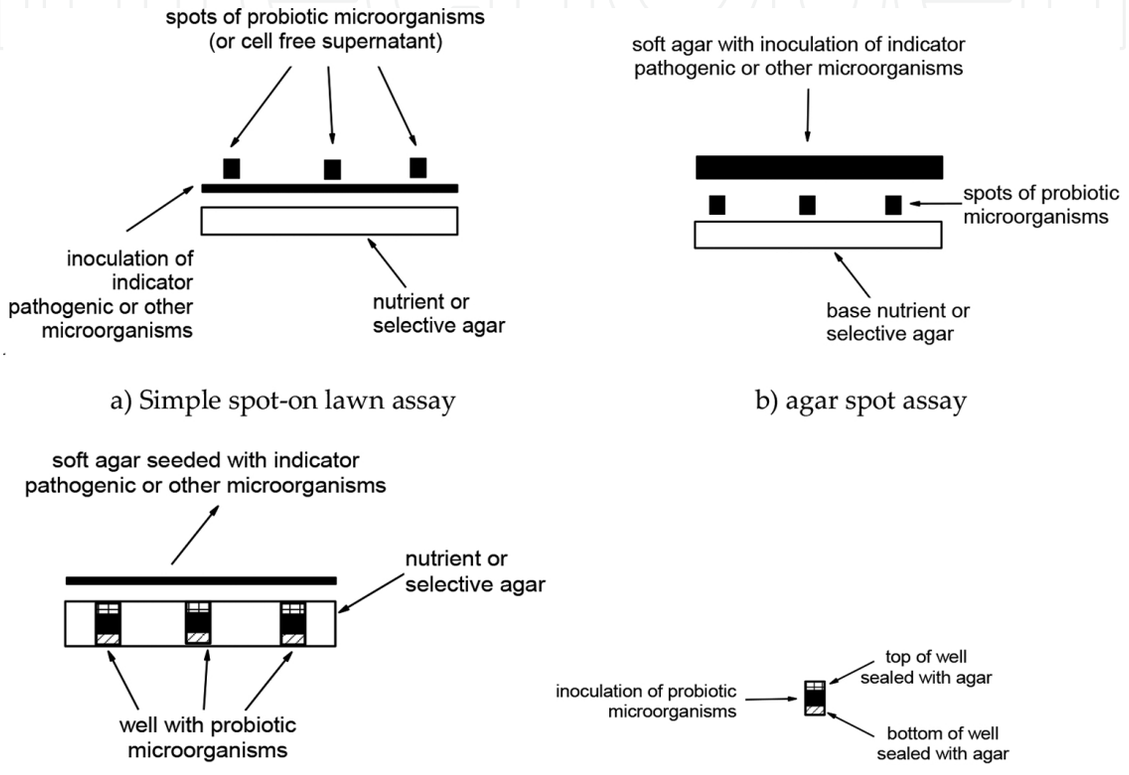


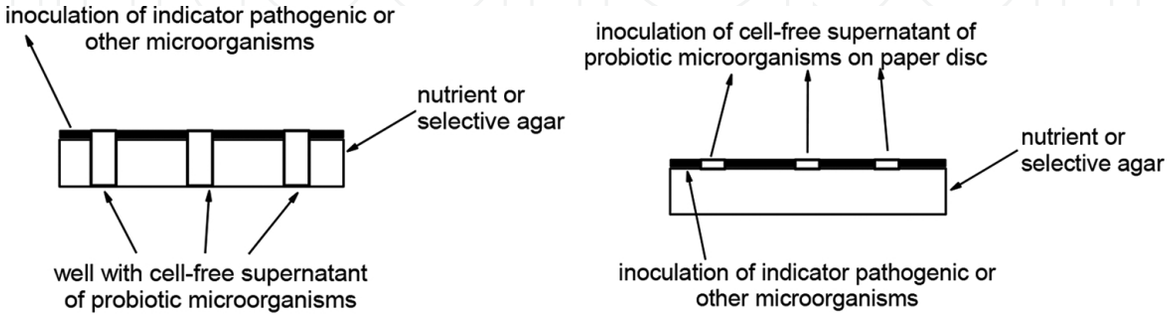
Figure 2. Schemes of different versions of spot-on lawn/agar spot assays: (a) simple spot-on lawn assay, (b) agar spot assay and (c) spot-on lawn assay with wells.

The fourth modification is the cross streak assay [26] where each probiotic strain is streaked in three parallel lines onto agar using a 1-mL loop. Once these lines have dried, test pathogenic strains are streaked perpendicular to these initial strains in the same fashion, giving three possible zones of inhibition for each combination of strains. It was assumed that when there is inhibition, it is caused by the tester probiotic strain hindering the growth of the second-streaked (indicator) strain.

3.2.2. Agar well diffusion assay/paper disc assay

The agar well diffusion assay (AWDA) (**Figure 3a**) is used for determining the antagonistic effects of cell-free supernatants. The general principle of agar well diffusion assay consists of

the following steps: different nutrients, selective or differential media, are prepared. The plates are inoculated with the chosen indicator microorganism. The 6-mm or 7-mm wells are bored in each plate. Aliquots of different dilutions of cell-free supernatants are pipetted into the wells. After incubation, the antimicrobial activity is expressed either as inhibition zone or as arbitrary units (AU/mL) [20, 22]. The paper disc assay (**Figure 3b**) is a modification where instead of making wells, discs measuring 6 mm are absorbed with aliquots of cell-free supernatant and placed on the agar inoculated with indicator strains. After incubation, the inhibition zone is evaluated based on the clear zone around the paper disc [23].



a) Agar well diffusion assay b) Paper disc assay

Figure 3. Scheme of the agar well diffusion assay and paper disc assay: (a) agar well diffusion assay and (b) paper disc assay.

3.2.3. Co-culturing assays for determining the antimicrobial activity

Determining the antimicrobial activity of probiotics against common pathogens is also possible with the co-culturing assay. This method includes the following steps: preparation of incubation media (i.e., nutrient broth, reconstituted skim milk, sterilized milk, yogurt, whey, etc.). Aliquots of pathogenic and probiotics microorganisms are inoculated into the incubation media. The samples are mixed well and incubated. After incubation, the population of pathogenic bacteria are counted on appropriate agars. Values are usually expressed as log cfu/mL [14–16, 27, 28].

The microtitre plate assay is a version of the co-culturing assay that includes the following steps: cell-free supernatant of active probiotic or other investigated microorganism is prepared and divided into several parts that undergo different conditions (i.e., NaOH added to neutralize pH, left acidic, heated, etc.). Pathogenic microorganisms are cultured and added to appropriate broth. The microtitre plate is used to prepare mixes of probiotics/cell-free supernatants and pathogenic microorganisms and incubated at suitable incubation conditions. Before and after incubation, the optical density at 620 nm is measured and the suppressive activity is calculated as a percentage of inhibition of pathogen growth [15].

Another important type of co-culturing assay is using cell lines. As several important mechanisms underlying the beneficial effects of probiotics include the effects of probiotic properties on specific tissues, particularly on the intestine, the evaluation of probiotic effects on human

intestinal cell lines *in vitro* is meaningful as these cells mimic the systemic environment of an organism and are used as a biological matrix alternative to *in vivo* tests. In fact, *in vitro* evidence is particularly important considering that the EU directives tend to discourage *in vivo* experiments on animals [29]. Several different cell lines have been used, such as HT-29 cell line from human colon [29], IPEC-J2 porcine neonatal jejunal cell line [30], Vero African green monkey kidney epithelial cell line [31], Caco-2 colon adenocarcinoma cell line [32], HIEC-6 normal epithelial small intestine cell line [33], BALB/c3T3 murine embryonal fibroblast cell line [29] and many others. Cells are routinely grown in Dubelco's modified Eagles' medium (DMEM), McCoy's 5a medium or other medium and seeded in well plates or microtitre plates that are incubated at 37°C for 24 h in 1 mL medium in 5% CO_2. Probiotic and pathogenic microorganisms are then added and the cell viability is determined after incubation.

3.3. *In vivo* methods for determining the antimicrobial/antagonistic effect of probiotics against other microorganisms

For *in vivo* testing, randomized double blind, placebo-controlled human trials should be undertaken to establish the efficacy of the probiotic product. The consultation recognized that there is a need for human studies in which adequate numbers of subjects are enrolled to achieve statistical significance. In order to ascertain that a given probiotic can prevent or treat a specific pathogen infection, a clinical study must be designed to verify exposure to the said pathogen (preventive study) or that the infecting microorganism is that specific pathogen (treatment study). If the goal is to apply probiotics in general to prevent or treat a number of infectious gastroenteritis or urogenital conditions, the study design must define the clinical presentation, symptoms and signs of infection, and include appropriate controls [4].

However, many scientists have reverted to *in vivo* animal studies. The animal models do not necessarily provide scientifically appropriate and relevant results for human, due to obvious species-specific differences in anatomy, biochemistry, physiology, pharmacokinetics and toxic responses. Especially, in medicine and pharmacy, the "safety testing" on animals led to thousands of deaths worldwide due to the evidence that animal tests are not only worthless, but they are also dangerously unpredictable. The use of replacement alternative methods, especially incorporating human cells and tissues, avoids such confounding variables [32].

The European legislation OJ L136 of 08.06.2000 includes the 3Rs regulation that results in important reduction of studies on animal models and consists of the following. *Reduction*: using alternative methods for obtaining comparable levels of information from the use of fewer animals in scientific procedure or for obtaining more information from the same number of animals. *Refinement*: using alternatives methods that alleviate or minimise potential pain, suffering and distress, and which enhance animal wellbeing. *Replacement*: using alternative methods that permit a given purpose to be achieved without conducting experiments or other scientific procedures on animals [32].

The *in vivo* animal model antimicrobial study is described as follows: briefly, all animal models include at least two groups under controlled settings. One group receives chosen probiotic and pathogen (treated infected group) and the other receives only the pathogen (untreated infected group). The observed difference includes the exanimation of faeces as well as the examination

of different cells after scarifying the animals (spleens, lymph nodes, blood, liver, colon, cecum, etc.). Animals used in these studies include mice, rats, chicks, rabbits, pigs, Fish and even worms [30, 34–37]. Work is done under accordance with the guidelines of the European convention for the protection of vertebrate animals used for experimental and other scientific purposes (Directive 86/609/EEC).

4. Recent results of *in vitro* antimicrobial/antagonistic assays for various probiotic strains

The following section contains results of the antimicrobial/antagonistic assays for various probiotic strains or strains with probiotic-like properties against various potential pathogens, spoilage microorganisms, or other probiotic microorganisms. The results reported using different assays (spot-on lawn/agar spot, agar well diffusion/paper disc, co-culturing, microtitre plate and cell line assays) are published by various authors stated in the text and some of the individual procedures are briefly explained.

4.1. Recent results of selected spot-on lawn/agar spot antimicrobial assays

The antimicrobial activity of *Lactobacillus plantarum* EM against seven potential pathogens using the spot-on-lawn assay was conducted by Choi and Chang [10]. The following nutrient and selective media were used: Luria-Bertani agar for *E. coli* O157:H7 ATCC 43895, *P. aeruginosa* ATCC 27853, *S. enterica* serovar Typhi ATCC 19430, nutrient agar supplemented with 2% NaCl for *V. parahaemolyticus* ATCC 17802 and tryptic soy agar for *B. cereus* KCTC 3624, *M. luteus* ATCC 15307 and *S. aureus* ATCC 29123. All potential pathogenic bacteria had an initial inoculum of 6 log cfu/mL. An aliquot of 10 μL of *L. plantarum* EM was spotted onto each plate. The plates were incubated aerobically at 37°C. After incubation the arbitrary units (AU/mL) were determined. *L. plantarum* EM exhibited strong antimicrobial activity against the seven chosen potential pathogenic bacteria. The strongest activity was noted against *V. parahaemolyticus*, ATCC 17802 (25600 units) and the weakest activity was noted for *S. aureus*, ATCC 29123 (200 units). As one of the most important requirements of probiotics is a broad antimicrobial spectrum, the authors found that *L. plantarum* EM fulfilled the beneficial requirements of probiotics [10].

In another research [24], screening for bacteriocins using the spot-on lawn method was used. One hundred and fifty lactic acid bacteria were isolated from samples of traditional fermented Vietnamese pork. The isolate named *L. plantarum* KL-1 was found to produce bacteriocins, effective against various Gram-positive and Gram-negative bacteria. The test was conducted by preparing two layers of soft agar (0.8% agar). The first layers were poured into the plate; then the top layer, which included 5 mL of soft agar together with 10 μL (about 10^7 cfu/mL) of freshly grown test bacterial strains, was added. The Gram-positive strains included *S. aureus* TISTR 118, *E. faecalis* JCM 5803, *E. faecalis* TISTR 888, *L. lactis* subsp. *cremoris* TISTR1344, *L. mesenteroides* JCM 6124, *L. mesenteroides* TISTR 942, *L. sakei* subsp. *sakei* JCM 1157, *L. sakei* TISTR 890, *L. plantarum* ATCC 14917, *L. inoccua* ATCC 33090, *Streptococcus* sp. TISTR 1030, *B. coagu-*

lans JCM 2257, *B. coagulans* TISTR 1447 and *B. campeatris* NBRC 11547. The Gram-negative test strains included *P. fluorescens* TISTR 358, *P. fluorescens* JCM 5963 and A. *hydrophila* TISTR 1321. Bacteriocin activity was tested by spotting 10 μL of previously prepared cell-free supernatant (in different dilutions) of the isolate *L. plantarum* KL-1. The inhibition zone was observed after overnight incubation at the proper temperature for each indicator microorganisms and the spectrum was expressed at arbitrary units (AU) [22]. The results show that the antimicrobial activity of the bacteriocin was strain specific. The bacteriocin was most effective against both strains of *L. sakei*. The bacteriocin was less effective against certain Gram-positive bacteria (both strains of *E. faecalis*, *L. plantarum* and both strains of *L. mesenteroides* subsp. *mesenteroides*). However, it was not effective against *S. aureus*, both strains of *B. coagulans* and any of the chosen Gram-negative bacteria.

Tharmaraj and Shah [16] used the spot-on-lawn technique with wells to test the inhibition of chosen pathogenic bacteria (*E. coli*, *S. typhimurium*, *S. aureus* and *B. cereus*) and spoilage bacteria (*B. stearothermophilus* and *P. aeruginosa*) by one strain of *L. casei* (Shirota YLC); two strains of *L. paracasei* subsp. *paracasei* (LCS1, LC01), *L. acidophilus* (LA5, LAC1) and *B. animalis* (BB12, BLC1); three strains of *P. freudenreichii* subsp. *shermanii* (P, PS1, PB10360) and four strains of *L. rhamnosus* (LC705, LBA, LGG, LR1524). MRS agar was used for lactobacilli (*L. acidophilus*, *L. casei*, *L. paracasei* subsp. *paracasei* and *L. rhamnosus*), MRS agar + L-cysteine (0.05%) for *B. animalis*, NaLa agar for *P. freudenreichii* subsp. *shermanii* and nutrient agar was used for pathogenic bacteria. Suitable agar (25 mL) was poured into the plates and wells were cut with a sterile metal borer. The bottom of the wells was sealed with 0.8% agar. Active culture (50 μL) of producing probiotics was then filled into the wells and left at room temperature for 2 h, followed by incubating at 37°C for 3 h. The remaining depth of the well was sealed with 1% agar. Finally, the spotted plates were overlaid with 10 mL of 0.8% agar seeded with about 10^7 cfu/mL of pathogenic bacteria. The plates were incubated anaerobically for 72 h at 37°C for all chosen pathogenic and spoilage bacteria. Both chosen spoilage bacteria were additionally incubated aerobically for 24 h at 37°C. On average, among all the probiotic and spoilage bacterial interactions, the spore formers were inhibited by the probiotic microorganisms to a greater extent (average zone of inhibition, 19 mm) than the non-spore formers (average zone of inhibition, 14 mm). Also, the Gram-positive bacteria (average zone of inhibition, 18 mm) were inhibited more than the Gram-negative bacteria (average zone of inhibition, 14 mm). Strains of *P. freudenreichii* subsp. *shermanii* did not show notable inhibitory effect.

Soomro et al. [23] also used the spot-on lawn method to determine antimicrobial activity of various *Lactobacillus* species. They found that *L. acidophilus* J1 showed an inhibitory effect against *E. coli*. The plates were prepared by inoculating 100 μL of indicator strain *E. coli* grown in broth with 3.5 mL soft MRS agar and were overlaid over MRS agar. The plates were incubated for 2 h at 37°C after which 30 μL of cell-free supernatant of *L. acidophilus* J1 was spotted onto the overlaid surface. The pH of the cell-free supernatant was adjusted to 5.5 to eliminate the effect of organic acids and hydrogen peroxide. The plates were incubated at 37°C for 18 h and were subsequently examined for inhibition zones. It was found that the inhibitory effect against *E. coli* was due to the production of a bacteriocin.

Probiotic strains or strains with probiotic potential with efficient antimicrobial activity *in vitro*	**Indicator pathogenic microorganisms**	**Reference**
Lb:*L. casei* C1; *L. plantarum* C4	**G+:***L. monocytogenes* **G-:***S.* Typhimurium CECT 4157, *Y. enterocolitica* IP383	[35]
Lb:*Lactobacillus* MSMC64-1	**G+:** MRSA DMST 20651, 20654, **G-:** *S.* Typhi DMST 5784, *V. parahaemolyticus* DMST 5665, *S. dysenteriae* DMST 15111	[38]
Oth:*S.cerevisiae* JCM7255	**G+:***S. agalactiae*	[39]
Oth:*B. pumilus* B16, *B. mojavensis* J7	**G-:***V. parahaemolyticus*	[40]
Lb:*L. acidophilus* La-5, **Bb:** *B. longum* ATCC 15707	**G+:***S. aureus, L. monocytogenes;***G-:***E. coli* O157:H7	[41]
Lb:*L. acidophilus* JN188382, *L. fermentum* JN188383, *L. fermentum* JN188384, *L. buchneri* JN188385, *L. buchneri* JN188386, *L. buchneri* JN188387, *L. casei* JN188388, *L. casei* JN188389, *L. casei* JN188390	**G+:***E. faecium* ATCC 51558, *S. epidermidis* ATCC *12228*, *P. acnes ATCC 6919, L. monocytogenes, S. aureus* S244; **G-:** *E. coli ATCC 29181, K. pneumoniae* K36, *E. cloacae, S. sonnei ATCC 25931, H. pylori ATCC 43579, V. parahaemolyticus,* **fng:** *C. albicans* ATCC 44831	[42]
Oth:*E. faecium* CV1, LPP29, *W. cibaria* P71, *L. lactis* subsp. *cremoris* SMF110, *Lc. mesenteroides* subsp. *cremoris* SMM69, *P. pentosaceus* SMM73, TPP3	**G-:***T. maritimum* NCIMB2154, LL01.8.3.8, *V. splendidus* CECT528, DMC-1	[43]
Lb:*L. plantarum* P6	**G+:***S. aureus ATCC 25923, B. cereus, L. ivanovii ATCC 19119;***G-:***E. coli ATCC 25921, S. enterica, P. aeruginosa*	[44]
Oth:*E. faecalis* AP-216, *E. faecalis* AP 45	**G+:***C. perfringens* KCTC 3269, KCTC 5100, *L. monocytogenes* KCTC 3569, 3586, 3710	[45]
Oth:*B. subtilis* DCU, *B. pumilus* BP, *B. cereus* HL7	**G-:***V. parahaemolyticus*	[46]
Lb: LAB 18, LAB 48	**G-:***S. enterica* serovar Enteritidis phage type 13A, *E. coli* O157:H7, *C. jejuni*	[47]
Lb:*L. plantarum* CK06, CK19, B01, B07, K09, K10, K21, LM11, ZS07, ZS11 and ZS15	**G+:***S. aureus SSV25, S. epidermidis SSV30, S. lentus CCM 3472, E. faecalis* V583, *L. monocytogenes* CCM *4699,* **G-:** *A. calcoaceticus* CCM *4503; S. paucimobilis* CCM *3293; S. enterica subsp. enterica* TA100 CCM 3812	[48]
Lb:*L. fermentum* PXN 44, *L. plantarum* PXN 47	**G+:***E. faecalis* NCTC 00775; **G-:** *E. coli* NCTC 9001	[49]
Lb:*L. paraplantarum* FT259	**G+:***L. monocytogenes* IAL 633, *L. innocua* ATCC 3309	[50]

Where probiotics are divided as follows: lb: lactobacilli; bb: Bifidobacterium; oth: other; and pathogens are divided as follows: G+: Gram positive; G−: Gram negative; fng: fungi.

Table 1. A selection of assays published since 2013 of successful antimicrobial activity of chosen probiotics using the spot-on lawn/agar spot assay on chosen pathogenic microorganisms.

Assays published since 2013 of antimicrobial activity of chosen probiotics using the spot-on lawn/agar spot assay on chosen pathogenic microorganisms are noted in **Table 1**.

As noted in **Table 1**, the most common investigated probiotic strains or strains with probiotic potential were from the genus *Lactobacillus* (*L. plantarum, L. acidophilus, L casei* and *L. fermentum*). Several studies included probiotic strains of the genus *Bacillus*. The most common pathogens included in the assays were *S. aureus*, *L. monocytogenes*, *E. coli*, *Vibrio* spp. and *Salmonella* spp. One study examined the antimycotic properties of probiotics against various strains of the genus *Aspergillus*. Different strains of *E. faecium* were on the one hand used as probiotics and on the other hand used as potential pathogen, thus proving the dualistic nature of this species.

4.2. Recent results of selected agar well diffusion assays/paper disc methods

The agar well diffusion assay was conducted in the research by Ali et al. [51], where 14 isolates with probiotic potential were screened for antimicrobial activity against *E. coli* and *S. aureus*. The probiotic isolates were identified as two *Lactobacillus* spp. (S2a3 and S4b1), eleven *Bifidobacterium* spp. (FCb1, Kb2, LZa7, LZb8, RC1b8, RC2b4, RC4a3, RC4b2, SCa4, SCb2 and Y2a5) and one *Streptococcus* spp. (RC2b3). A volume of 100 μL of cell-free supernatant of isolates with probiotic potential was filled in 7-mm wells cut in nutrient agar previously inoculated with *E. coli* or *S. aureus*. The diameter of inhibition zone was measured after 48 h of incubation. The supernatant obtained from all 14 isolates exhibited varying degrees of inhibitory activity against *S. aureus* and *E. coli*. The isolates LZb8, S4b1 and RC4a3 exhibited the superior antibacterial activity with inhibition zones ranged 8.3–8.4 mm. The least activity was recorded for the isolates SCa4 and RC4b2 (inhibition zone ranged 2.3–2.5 mm), whereas the isolates Kb2, LZa7, RC2b4, RC2b3, SCb2 and Y2a5 (inhibition zone ranged 3.5–4.8 mm) were moderately active against *S. aureus*. It is worth mentioning that the inhibitory activity of the tested isolates supernatants was slightly less against *E. coli* as compared to that obtained against *S. aureus*, indicating that *E. coli* could be less sensitive.

In the previously mentioned research by Soomro et al. [23], the paper disc method was also used. Sterile filter discs measuring 6-mm diameter with an absorbed aliquot of 20 μL of cell-free supernatant of *L. acidophilus* J1 were placed on MRS and nutrient agar plates containing the target strain *E. coli*. After incubation at 37°C, the inhibitory activity was evaluated. It was found that the paper disc assay yielded an inhibition zone of 10 mm and was more appropriate compared to the spot-on lawn assay.

Assays published since 2013 of antimicrobial activity of chosen probiotics using the agar well diffusion assay or the paper disc assay on chosen pathogenic microorganisms are noted in **Table 2**. The results show that the agar well diffusion assay or the paper disc assay is the most common method used for determining the antimicrobial or antagonistic effect. The most common investigated probiotic strains or strains with probiotic potential were again from the genus *Lactobacillus*. Some assays included bifidobacteria and bacteria from the genus *Pediococcus* and *Lactococcus*. The most common pathogens included in the assays were

again *S. aureus*, *L. monocytogenes*, *E coli*, *Vibrio* spp., *Aeromonas* spp. *Salmonella* spp. and *Pseudomonas aeruginosa*. One study even assessed the antagonistic activity of probiotics against herpes simplex virus types 1 and 2 and one study investigated the antimicrobial activity of probiotics against the protozoa *Giardia lamblia*. However, using cell-free supernatants does not mimic real conditions. Therefore, further assays are necessary.

Probiotic strains or strains with probiotic potential with efficient antimicrobial activity *in vitro*	**Indicator pathogenic microorganisms**	**Reference**
Lb:*L. acidophilus* PBS066, *L. fermentum* PBS073, *L. plantarum* PBS067, *L. rhamnosus* PBS070, *L. reuteri* PBS072, **Bb:** *B. animalis* subsp. *lactis* PBS075, *B. longum* subsp. *longum* PBS108	**G+:***S. aureus* ATCC 6538, *E. faecalis* ATCC 29212, **G-:** *E. coli* ATCC 25922, *P. aeruginosa* ATCC 9027, **fng:** C. *albicans* ATCC 10231	[29]
Lb:*L. salivarius* JM41, JK21V, JM31, JS2A, JM14, JK22, JM2A1 and JM32, *L. plantarum PZ01* **Oth:** *P. acidilactici* JM241 and JH231, *P. pentosaceus* JS233, *E. faecium JS11*	**G+:***S. aureus* ATCC 29213, **G-:** *E. coli* K88, 25922 and 1569, *S. Enteritidis* ATCC 13076, *S*. Typhimurium ATCC 14082	[34]
Lb:*L. casei* C1, *L. plantarum* C4, *L. acidophilus*	**G+:***L. monocytogenes*,**G-:***E. coli* C17, *S. enterica* ser Typhimurium CECT4156, *Y. enterocolitica* IP383	[35]
Lb:*Lactobacillus* MSMC64-1	**G+:** MRSA DMST 20651, 20654, **G-:** *S.* Typhi DMST 5784, *V. parahaemolyticus* DMST 5665, *S. dysenteriae* DMST 15111	[38]
Lb:*L. acidophilus* La-5, **Bb:** *B. longum* ATCC15707	**G+:***S. aureus, L. monocytogenes*,**G-:***E. coli* O157:H7	[41]
Lb:*L. plantarum* WCFS1, *L. plantarum* NA7	**G+:***L. monocytogenes* CIP 81.3 ILSI NA 39, **G-:** *E. coli* O157:H7 ATCC 43888, *S. enterica* ser *Enteritidis* CIP 81.3	[52]
Lb:*L. casei* PTCC 1608, *L. rhamnosus* PTCC 1637	**G-:***P. aeruginosa* PTCC 1430	[53]
Oth:*B. amyloliqufaciens*	**G+:***C. difficile*	[54]
Oth:*P. pentosaceus* KID7	**G+:***S. aureus* KCCM1133515, MRSA CCM40510, *S. epidermidis* KCTC 191712, *L. monocytogenes* KACC1076420, *B. cereus* KACC11240 **G-:** *S.* Typhi KCTC2514, *S. choleraesuis* KCTC293215, *S. gallinarum* KCTC293126, *S. boydii* KACC10792 14, *Y. enterocolitica* KACC1532020, *E. coli* O138KCTC261511, O1KCTC2441, *P. aeruginosa* KCCM 1180218,	[55]
Lb:*L. rhamnosus* FM13, FM14, FM22, FS2, FS10, PS2, PS11, SF6, SP13, *L. paracasei* CM1, CM2, MF5, PM8	**G+:***S. aureus* ATCC 6538, *L. monocytogenes* DSM 12464, *E. faecalis*, **G-:** *E. coli* ATCC 25922	[56]
Lb: L. *casei* LC-01, *L. acidophilus* LA-5, *L. paracasei*	**fng:***A. niger* PTCC 5012, *A. flavus* PTCC 5004, *A. parasiticus* PTCC 5286, *P. chrysogenum* PTCC 5035	[57]

Probiotic strains or strains with probiotic potential with efficient antimicrobial activity *in vitro*	**Indicator pathogenic microorganisms**	**Reference**
Lb:*L. viridescens* NRRL B-1951	**G+:***L. monocytogenes* CWD 1002, CWD 1198	[58]
Lb:*L. plantarum* S2	**G+:***S. aureus* CMCC2607, **G-:** *E. coli* CMCC44825, *S.* Typhimurium CMCC50115, *S. flexneri* CMCC51061	[59]
Oth:*B. subtilis* JQ302302, *B. aerophilus* JQ312663	**G-:***A. hydrophila* ATCC 49140, MTCC 1739, *Aeromonas* sp. JX136697, JX136698, A. *enteropelogenes* JX136699, *P. rettgeri* JX 136696	[60]
Lb:*L. acidophilus* L-1, *L. bulgaricus* 6, *L. plantarum* 24-4B, *L. fermentum* 1, *L. brevis,* **Bb:** *B. animalis* subsp. *lactis* L-3	**G+:***S. aureus, B. cereus* **G-:***E. coli*	[61]
Lb:*L. plantarum* DK211, DK303; *L. paracasei* DK215, L. *sakei* DK301	**G+:***S. aureus* KCTC 3881, *E. faecalis* KCTC 2011, *B. cereus* KCTC 3624	[62]
Lb: L. *mesenteroides* subsp. *mesenteroides* SD1, SD23, SF2, SF3	**G+:***S. aureus* ATCC 25923, FRI 184, *L. monocytogenes* ATCC 19115, **G-:** *E. coli* ATCC43895, *S. enterica* ATCC 14028	[63]
Oth:*L. mesenteroides* MTCC 5442, *B. subtilis*	**G-:***V. cholerae*	[64]
Lb:*L. fermentum* M059, *L. fermentum* F-6, **Oth:** *W. cibaria* 4213	**G+:***S. aureus* ATCC 6538, *B. cereus* NCIM 245, *B. subtilis* ATCC 6633, **G-:** *E. coli* ATCC 25922, S. Typhi 25, *P. aeruginosa* ATCC 27853	[65]
Lb:*L. brevis* DT24	**G-:***E. coli* MTCC 729	[66]
Lb:*L. rhamnosus* (4 strains)	**G-:***Helicobacter pylori*	[67]
Lb:*L. plantarum, L. salivarius*	**G+:***S. aureus* ATCC 6538S, *C. perfringens* ATCC 13124, **G-:** *E. coli* ATCC 8734, *S. Enteritidis* ATCC 13311, *R. anatipestifer* ATCC 11845, *P. multocida* ATCC 43137	[68]
Lb:*L. acidophilus* P106, *L. plantarum* P164	**Prs:***Giardia lamblia*	[69]
Lb:*L. casei*	**G-:** S. *flexneri, S. sonnei*	[70]
Lb:*L. helveticus* PJ4, *L. plantarum* PJ7	**G+:***S. aureus* MTCC737, **G-:** *E. coli* MTCC443, *S.* Typhimurium MTCC733, *S. fleksneri* MTCC1457, *P. aeruginosa* MTCC1688	[71]
Lb:*L. salivarius* K35, K43	**G+:***S. mutans* ATCC 25175	[72]
Oth:*B. amyloliquefaciens* KATMIRA1933	**Vr:** Herpes simplex virus types 1 and 2	[73]
Oth:*B. amyloliquefaciens* FPTB16	**G-:***E. tarda, A. hydrophila, V. harveyi, V. parahaemolyticus*	[74]

Where probiotics are divided as follows: lb: lactobacilli; bb: Bifidobacterium; oth: other; and pathogens are divided as follows: G+: Gram positive; G−: Gram negative; fng: fungi; prs: parasite.

Table 2. A selection of assays published since 2013 of successful antimicrobial activity of chosen probiotics using the agar well diffusion assay or the paper disc assay on chosen pathogenic microorganisms.

4.3. Recent results of selected co-culturing assays

The influence of the potential pathogenic bacteria *P. aeruginosa* ATCC 27853 against various combinations of probiotic supplements and the kefir microbiota was investigated using the co-culturing method [6]. One multispecies, one oligospecies and one monospecies probiotic supplement as well as kefir microbiota from kefir grains originally from the Caucasian mountains were used. The multispecies supplement contained *L.* acidophilus (NIZO 3678; NIZO 3887), *L. paracasei* NIZO 3672, *L. plantarum* NIZO 3684, *L. rhamnosus* NIZO 3689, *L. salivarius* NIZO 3675, *B. bifidum* NIZO 3804, *B. lactis* NIZO 3680 and *E. faecium* NIZO 3886. The oligospecies supplement contained *L. acidophilus* LA-5, *B. infantis* BB-12 and *E. faecium.* The monospecies supplement contained *L. reuteri* DSM 17938. Co-culturing was conducted by adding 1 mL of probiotic samples and 1 mL of overnight *P. aeruginosa* suspension in 40 mL previously sterilized bovine milk. Samples were incubated for 4 days at 25°C with agitation (250/min). After incubation, serial 10-fold dilutions were prepared for all samples and the *P. aeruginosa* populations were enumerated on cetrimide agar with added glycerol. It was found that the *P. aeruginosa* population in milk without probiotics reached an average of 9.2 log/mL, whilst the *P. aeruginosa* population in milk reached 5.2, 8.3, 8.3 and 5.0 for the samples with the multispecies, oligospecies and monospecies probiotic supplement as well as the kefir microbiota respectively. This research thus found that the decrease of the potential pathogen *P. aeruginosa* was dependent on the type of probiotic microbiota and that both multispecies microbiota (multispecies probiotic supplement kefir microbiota) with a much more diverse population than the other two samples (oligospecies probiotic supplement and the monospecies supplement) exhibited an efficient synergistic effect [7]. Both samples also exhibited a higher increase in the total population of anaerobic microorganisms after fermentation with *P. aeruginosa* thus indicating that a more successful quorum-sensing regulatory network was established and yielded antagonistic effects against the potential pathogen *P. aeruginosa*.

Tharmaraj and Shah [16], as already mentioned in the previous section, also investigated the inhibition effect of various probiotics against chosen pathogenic and spoilage bacteria with the co-culturing method. Briefly, 9 mL of reconstituted skim milk was inoculated with 1 mL of overnight culture of probiotic bacteria and 0.1 mL of pathogenic or spoilage bacteria. The medium was mixed well and incubated at 37°C for 24 h. / were counted on nutrient agar and the log population calculated (**Table 6**). All four pathogenic bacteria were inhibited by all probiotic strains tested to varying degrees. On average, the probiotic bacteria reduced the population of pathogenic bacteria by 2.8 log units. *B. cereus* was inhibited to a greater degree by all probiotic bacteria and strains than other pathogenic bacteria. On average, the inhibitory effect of all probiotic bacteria and strains was the weakest against *E. coli*. *S. aureus* was inhibited to a greater degree by *B. animalis* and *L. rhamnosus* than the other probiotic bacteria.

Ratsep et al. [15] published their research of a microtitre plate assay on the antimicrobial effect of *L. plantarum* (five strains: N11, N27, N33, N44 and E) supernatant against various *C. difficile* strains (six clinical isolates from Norwegian patients, six clinical isolates from Estonian patients and two reference strains: VPI 10463 and M13042). Overnight *C. difficile* cultures were added to BHI broth with a density according to McFarland 3.0. Various reaction mixes were prepared (natural (acidic), neutral (pH 6.0) and neutral, heated for 20 min at 100°C) and incubat-

ed under anaerobic conditions for 48 h at 37°C. The optical density at 620 mm was measured at the beginning of incubation and after 48 h and the suppressive activity of *L. plantarum* strains calculated as a percentage of inhibition. The highest inhibitions of *C. difficile* growth were in the samples of heated neutralized supernatants and the lowest in cases of the neutralized samples. There was statistically higher inhibition in heated neutralized supernatants versus neutralized supernatants of N11 and E56 lactobacilli strains. When comparing antagonistic activity of *L. plantarum* strains, there was a relevant difference only between N11 and N33 strains in the samples of heated neutralized supernatants. The neutralization of supernatant did not reduce its inhibitory effect. Thus, lowering the pH of the environment is not the main mechanism in inhibition of *C. difficile* by lactobacilli. Also heating of supernatant did not reduce its activity; thus, some thermostable compounds may be involved in the inhibition. The above-mentioned authors [15] also performed the co-culturing assay with the *L. plantarum* strains and *C. difficile* strains by inoculating 50 mL of brain heart infusion broth with 50 μL of lactobacilli suspension and 50 μL of *C. difficile* suspension and incubating under anaerobic conditions for 48 h at 37°C. After incubation, serial 10-fold dilutions were prepared and the *C. difficile* populations were enumerated on fastidious anaerobe agar. It was found that the five *L. plantarum* strains (N11, N27, N33, N44 and E) were able to inhibit the growth of the 14 *C. difficile* strains (six clinical isolates from Norwegian patients and Estonian patients and two reference strains: VPI 10463, M13042) in the co-culture incubation as the average log cfu/mL after 48 h was 3.0, whereas the average log cfu/mL of the *C. difficile* strains alone was 7.0.

Another important co-culturing method is the use of cell lines as noted in the research by Abdel et al. [31] of 12 lactobacilli isolates interfering with the adherence and invasion of *S.* Typhi 66 using kidney epithelial cell line *Vero* (ATCC CCl-81). The same authors also investigated this interference with the co-culturing assay in MRS broth. It was found that nine lactobacilli isolates inhibited the growth of *S.* Typhi in the co-culturing assay. Nine lactobacilli isolates were also successful in achieving a >50% inhibition of adherence of *S.* Typhi isolate (SS6) to Vero cells.

Assays published since 2013 of antimicrobial activity of chosen probiotics using various co-culturing assays on chosen pathogenic microorganisms are noted in **Table 3**. The results were similar to the results of spot-on lawn and agar well diffuse assays.

Probiotic strains or strains with probiotic potential with efficient antimicrobial activity *in vitro*	**Indicator pathogenic microorganisms**	**Reference**
Lb:*L. plantarum* FH185	**G+:***S. aureus*,**G-:***S.* Typhimurium	[9]
Lb:*L. acidophilus* La-5, **Bb:** *B. longum* ATCC15707	**G+:***S. aureus, L. monocytogenes*,**G-:***E. coli* O157:H7	[41]
Lb:*L. acidophilus* P106, *L. plantarum* P164	**Prs:***Giardia lamblia*	[69]
Lb:*L. rhamnosus* GR-1, *L. reuteri* RC-14	**Fng:***C. glabrata*	[75]
Lb:*L. helveticus* KLDS 1.8701	**G+:***S. aureus* ATCC 25923, *L. monocytogenes* ATCC 19115, **G-:** *E. coli* O157:H7 ATCC 43889, *S.* Typhimurium ATCC 14028	[76]

Probiotic strains or strains with probiotic potential with efficient antimicrobial activity *in vitro*	Indicator pathogenic microorganisms	Reference
Lb:*L. reuteri***Oth:***B. subtilis* MA139	**G-:***E. coli* K88	[77]
Lb:*L. plantarum* C014	**G-:***A. hydrophila* TISTR 1321	[78]
Lb:*L. acidophilus*, **Oth:***Pediococcus*	**G-:***S.* Enteritidis 13A	[79]
Lb:*L. paracasei* CNCM I-4034, *L. rhamnosus* CNCM I-4036, **Bb:** *B. breve* CNCM I-4035	**G-:***E. coli* ETEC CECT 501, *S.* Typhimurium CECT 443 *S.* Typhi CECT 725, *S. sonnei* CECT 457	[80]
Lb:*L. fermentum* 907, **Bb:** *B. longum* 1011	**G-:***E. coli* O157:H7, *E. coli* O86	[81]

Where probiotics are divided as follows: lb: lactobacilli; bb: Bifidobacterium; and pathogens are divided as follows: G+: Gram positive; G−: Gram negative; fng: fungi; prs: parasite.

Table 3. A selection of assays published since 2013 of successful antimicrobial activity of chosen probiotics using the co-culturing assay on chosen pathogenic microorganisms.

5. Recent results of *in vivo* antimicrobial/antagonistic assays for various probiotic strains

5.1. Recent results of determining the *in vivo* antimicrobial assays using animal models

In the research by Mazaya et al. [36], both *in vitro* and *in vivo* studies were conducted. The significant *in vitro* antimicrobial activity (no method specified) of two lactobacillus strains isolated from Egyptian dairy products (*L. plantarum* LA5 and *L. paracasei* LA7) was found against several potential pathogens: *S. aureus* ATCC 25923, *B. subtilis* ATCC 23857, *M. luteus* ATCC 21882, *P. aeruginosa* ATCC 27853 and *S.* Typhi. *In vivo* assays were also conducted on 5-week-old male mice, divided into six groups (10 mice/group). Animals within different treatment groups were treated daily for 8 days as follows: Group 1, untreated control; Group 2, animals challenged with single inoculation *S.* Typhi (200 μL aliquot of 1X 108/P.O); Group 3, animals treated orally with *L. plantarum* (LA5) (200 μL aliquot of 1X 108/P.O) for 7 days; Group 4, animals treated orally with *L. paracasei* (LA7) (200 μL aliquot of 1X 108/P.O) for 7 days; Group 5, animals challenged with single inoculation *S.* Typhi, then treated with LA5 for next 7 days; Group 6, animals challenged with single inoculation *S.* Typhi and treated with LA 7 for next 7 days. Administration of LA5 or LA7 counteracted the pathogenic effect resulting from Salmonella infection. The lactobacilli succeeded to get rid of salmonellosis based on its phagocytic and immunostimulant activity against typhoid antigen.

Lazarenko et al. [82] conducted an *in vitro* and *in vivo* assay to determine anti-staphylococcal actions of certain probiotic cultures (*L. casei* IMV B-7280, *L. acidophilus* IMV B-7279; *B. longum* VK1 and *B. bifidum* VK2). *In vitro* assay using perpendicular strokes yielded antagonistic activity against all three strains of *S. aureus* (209-P, 43, 8325-4). The *in vivo* study with *S. aureus* 8325-4 on mice showed that the combination of probiotics (*L. casei* IMV B-7280, *B. longum* VK1 and *B. bifidum* VK2) was most successful as after day 9 no colonies of *S. aureus* 8325-4 were found in the vagina of the mice.

In the study by Bujalance et al. [35], a lack of correlation between *in vitro* and *in vivo* methods of the antimicrobial activity of probiotic lactobacilli against enteropathogenic bacteria was determined. In this study, the *in vitro* assay using the agar spot test showed that 20 strains of probiotic lactobacilli successfully inhibited *Y. enterocolitica*, *S. enterica* ser Typhimurium and *L. monocytogenes*. However, in the *in vivo* study using mouse models the selected strains (*L. casei* C1 and *L. plantarum* C4) lacked protective effects against *S.* Typhimurium. Similar conclusions of finding no antagonism *in vivo* are noted in the study by Bratz et al. [83]. Another study [79] found that the tea Yerba mate exhibited antagonistic activity *in vitro* but as a feed additive did not reduce S. Enteritidis colonization *in vivo* in broiler chickens. These studies prove that successful *in vitro* assays do not necessarily mean that the chosen microorganism with probiotic properties will be successful in real conditions. On the other hand, *in vivo* animal studies do not automatically prove antagonism in humans or other species. Therefore, the justification of using vertebrate animal models is questionable [32]. Gupta et al. [84] used *Drosophila melanogaster* commonly known as the "fruit fly," instead of a vertebrate animal model. It is a eukaryotic organism and is considered an alternative in the drug discovery process, mainly because the key physiological processes are well conserved from fly to humans. Moreover, a short life cycle, distinct developmental stages, easy cultivation, numerous offspring and a strong cytogenetic/genetic background make *Drosophila* a model organism to study many biological processes including toxicity testing. Zhou et al. [30] used porcine neonatal jejunal epithelial cell lines (IPEC-J2) for *in vitro* assay and worms (*Caenorhabditis elegans*) for *in vivo* testing of lactobacilli isolates against enterotoxigenic *E. coli*

Probiotic strains or strains with probiotic potential with efficient antimicrobial activity *in vivo* using animal models	**Indicator pathogenic microorganisms**	**Reference**
Lb:*L. reuteri* CL9, K16, K67 and S33	**G-:***E. coli* O149: K88 JG280	[30]
Lb:*L. salivarius* JM32, *L. plantarum* PZ01 **Oth:** *P. acidilactici* JH231	**G-:***S. Enteritidis* ATCC 13076	[34]
Lb:*L. plantarum* LA5 and *L. paracasei* LA7	**G-:***S.* Typhi	[36]
Oth:*B. amyloliqufaciens*	**G+:***C. difficile*	[54]
Lb:*L. plantarum* C014	**G-:***A. hydrophila* TISTR 1321	[78]
Lb:*L. casei* B–7280, **Bb:** *B. longum* VK1, *B. bifidum*VK2	**G+:** *S. aureus*8325–4	[82]
Lb:*L. plantarum* LR/14	**Ins:***Drosophila melanogaster*	[84]
Lb:*L. acidophilus* CH1	**Prs:***Enterocytozoon bieneusi*	[85]
Oth:*Pseudoalteromonas* sp.	**G-:***V. harveryi* ATCC 14126	[86]

Where probiotics are divided as follows: lb: lactobacilli; bb: Bifidobacterium; oth: other; and pathogens are divided as follows: G+: Gram positive; G−: Gram negative; fng: fungi; prs: parasite; ins: insect.

Table 4. A selection of assays published since 2013 of successful antimicrobial activity of chosen probiotics using the *in vivo* animal models on chosen pathogenic microorganisms.

The most recent *in vivo* antagonistic assays using animal models are noted in **Table 4**. These results confirm the strain specific antagonistic activity of chosen probiotics.

5.2. Recent results of determining the *in vivo* antimicrobial assays using clinical trials

Most important research on the antagonistic effect of probiotics are clinical trials, however only a few well conducted clinical studies have been reported. Most clinical studies include the comparison of antibiotic therapy with adjuvant probiotic therapy. In the study by Dore et al. [87], in this prospective, single centre, open label pilot study, patients scheduled for upper endoscopy for any reason and found to be positive for *H. pylori* infection were invited to enter. The intervention consisted of *L. reuteri* (DSM 17938, Reuflor, BioGaia AB, Sweden) 10^8 cfu/tablet plus pantoprazole (proton pump inhibitor) 20 mg twice a day. A 76% decrease in urease activity was observed. The absence of a control group with pantoprazole without *L. reuteri* however prevents any definite conclusion.

In the study by Pendharkar et al. [88] the clinical outcome for women conventionally treated for bacterial vaginosis and yeast infection with probiotics bacilli was investigated. This study is an example of the antibiotic therapy with adjuvant probiotic therapy. In the clinical trial, women were recruited in three groups as follows: women with bacterial vaginosis receiving clindamycin and metronidazole treatment together with a prolonged administration of EcoVag® (containing *L. rhamnosus* DSM 14870 and *L. gasseri* DSM 14869) for 10 consecutive days after each antibiotic treatment followed by weekly administration of capsules for next four months, women with recurrent vulvovaginal candidiasis receiving extended fluconazole and EcoVag® treatment, and women receiving extended fluconazole treatments only. The 6- and 12-month cure rates for bacterial vaginosis were 67 %. The 6- and 12-month cure rates for vulvovaginal candidiasis were 100 and 89% in women receiving fluconazole and EcoVag®, and 100 and 70% in women receiving fluconazole only. The study suggests that the treatment with antibiotics or anti-fungal medication in combination with EcoVag® capsules provide long-term cure against bacterial vaginosis and R-VVC.

Some of the most recent *in vivo* antagonistic clinical trials are noted in **Table 5**. These results confirm that adjuvant therapy with antibiotics and chosen probiotics enhances the antagonistic activity.

Probiotic strains with efficient antimicrobial activity using *in vivo* clinical trials and other therapies	**Pathogenic microorganisms or treated disease**	**Basic therapy**	**Type of trial**	**Reference**
Lb:*L. reuteri* DSM 17938	**G+:***H. pylori*	Proton pump inhibitor: pantoprazole	CT	[87]
Lb:*L. rhamnosus* DSM 14870, *L. gasseri* DSM 14869	**Bc:** not specified, **Fng**: *Candida* spp.	Antibiotics: Clindamycin, metronidazole, fluconazole	CT	[88]
Lb:*L. rhamnosus* GG (LGG), **Bb:** *B. bacterium* BB-12	**G+:***H. pylori*	Standard triple *H. pylori* eradication therapy with	DBRCT	[89]

Probiotic strains with efficient antimicrobial activity using *in vivo* clinical trials and other therapies	Pathogenic microorganisms or treated disease	Basic therapy	Type of trial	Reference
		antibiotics: omeprazole, pantoprazole, clarithromycin, amoxicillin, or metronidazole		
Lb:*L. rhamnosus* GG (LGG), *L. acidophilus* La-5, **Bb:** *B. bacterium* BB-12	**Dis:** AAD	Antibiotics: broad spectrum oral antibiotics	DBRCT	[90]
Lb:*L. acidophilus, L. paracasei***Bb:***B. lactis* **At:** bismuth	**G+:***H. pylori*	Antibiotics: broad spectrum oral antibiotics: lansoprazole, amoxicillin, clarithromycin	BDRCT	[91]
Lb:*L. casei* DN-114001	**Dis:** AAD	Antibiotics: broad spectrum oral antibiotics	CT	[92]
Lb:*L. acidophilus, l. rhamnosus, L. acidophilus, L. bulgaricus*, **Bb:***B. bifidus***Oth:***S. Thermophilus*	**Dis:** bacterial vaginosis	Antibiotic: metronidazole	CT	[93]

Where probiotics are divided as follows: lb: lactobacilli; bb: Bifidobacterium; oth: other; pathogens are divided as follows: bc: bacteria; G+: Gram positive; G−: Gram negative; fng: fungi; and clinical trials are divided as follows: DBRCT: double-blinded randomized clinical trial; CT: clinical trial; AT: additional therapy; Dis: disease; AAD: antibiotic associated diarrhoea.

Table 5. A selection of published since 2013 of antimicrobial activity of chosen probiotics using *in vivo* clinical trials on chosen pathogenic microorganisms or treatment of diseases.

6. Discussion and conclusions

The antimicrobial ability of probiotics is a very important trait and includes the production of antimicrobial compounds, competitive exclusion of pathogens, enhancement of the intestinal barrier function and others. Usually, probiotic strains produce more than one antimicrobial substance that may act synergistically, increasing the spectrum of targeted microorganisms. This property may be desirable as long as this antimicrobial spectrum is restricted to pathogenic microorganisms but it cannot be excluded that it will not affect the normal microbiota of the gut or other microbiotas as well [94]. The results show that probiotic properties are strain dependent and that strain identification is imperative [3].

Probiotic candidates have been accessed from very diverse habitats including faeces of breast-fed human infants [65, 69, 80, 85, 95], faeces of healthy adults [9, 15, 65, 70], faeces of elderly [81], faeces of children [25, 96], breast milk [42], human saliva [52], vaginal isolates of healthy women [66, 75], various fermented foods or beverages including raw or fermented milk [23, 35, 44], kefir [97], cheese [51, 56, 98], whey [99], yogurt [16, 41], dahi [100, 101], other dairy products [25, 36, 61], sourdough [102], sausages [17], fermented meat [24], kimchi [10, 62], maize [25, 59], fermented olives [103], Yerba mate [79], ragi [64], soy sauce [86], soil [104], as

well as animal origin including rat faeces [71], geese [68], calves [105], pigs [45], fish [39, 60, 63, 78] and other seafood [40, 43, 46] and many others.

By far, the most commonly investigated probiotic were bacteria of the genus *Lactobacillus* (*L. plantarum, L. acidophilus, L. fermentum, L. casei, L. paracasei* and *L. reuteri*). The genus *Bifidobacterium* and other probiotic microorganisms (*Lactococcus, Pediococcus, Enterococcus, Bacillus* and *Saccharomyces*) have been also been investigated, but to a somewhat lesser extent. Studies were also conducted on known probiotics from various tissue type collections. The most common pathogens used to test the antagonistic activity of probiotics were different strains of S. aureus, E. faecium, E. faecalis, L. monocytogenes, E. coli, various Salmonella, Vibrio and Yersinia spp., and P. aeruginosa.

The antimicrobial activity of probiotic microorganisms has a very wide area application including adjuvant therapy to antibiotic consumption or for correcting dysbiosis of the gastrointestinal tract microbiome due to diarrhoea [37, 38, 106], antagonistic activity in humans against urinary tract infections [26, 66, 75], eradicating *H. pylori* infections [87], nosocomial infections [15, 96], dental biofilm formation [72], lowering serum cholesterol [10, 55], treating fevers [31], as well as in the agro-food industry for manufacturing fermented products [44, 62, 107], preventing food spoilage [16, 23, 41], as food additives for functional foods [50, 56, 57, 59, 67, 97], as prophylactic agents, adjuvants or alternatives to antibiotic therapies to antibiotic therapy in poultry [34, 47, 68], cattle [105, 108], pigs [45], fish [74, 78] and other livestock industry [104], just to name a few.

The process of determining antimicrobial properties of probiotic is complex and includes *in vitro* assays, *in vivo* models or substitute models, clinical studies, metagenomic analyses and mathematical modelling. Only after all these steps are completed, a probiotic candidate can be identified as such [109]. *In vitro* studies are the most represented. Although, they are a crucial step in selecting probiotic candidates, they are only the first step as efficient antimicrobial activity via *in vitro* studies does not necessarily mean that the antimicrobial activity is present in *in vivo* assays. Therefore, further research methods (double-blinded randomized clinical trials) are necessary to prove the important antimicrobial trait of probiotic candidates. As noted in Section 5.2, there are only a few well-conducted published clinical studies. Most clinical studies include the comparison of antibiotic therapy with adjuvant probiotic therapy which is an important aim of probiotic consumption.

There is a clear need for more elaborate assays that would better represent the complex interactions between the probiotics and the host microbiome to understand the consequences of the *in situ* production of antimicrobials by the former [94]. Another important fact is that probiotics are often found to have higher antagonist activity as multispecies groups [6, 7, 26]. Quorum sensing among probiotics is also an important factor; however, quorum-sensing studies among probiotics are sparse. It is well known that microorganisms coordinate collective behaviour in response to environmental challenges using sophisticated intercellular communication networks and that they are not limited to communication within their own species but are capable of intercepting messages and coerce cohabitants into behavioural modifications [110], therefore probiotics are included. Although all these facts make re-

search of the antimicrobial/antagonistic activity of probiotics even more complex, it also presents a great opportunity for future research.

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