Antimicrobial activity of probiotic microorganisms: mechanisms of interaction and methods of examination

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FAO/WHO define probiotics as “live microorganisms which when administered in adequate amounts confer a health benefit to the host”. The administration of probiotics results in the prevention of a number of physical conditions and diseases and the prophylaxis and treatment of others. There are a number of requirements for probiotic strains in order to be included in the composition of probiotic preparations and probiotic foods. One of the requirements for probiotic strains is to exhibit antimicrobial activity against pathogenic, toxigenic and saprophytic microorganisms. The antimicrobial activity of probiotic microorganisms is strain-specific, but the mechanisms of interaction of probiotic and pathogenic microorganisms can be grouped into two groups: direct and indirect mechanisms. The direct mechanisms of interaction can be summarized in the following groups: 1) competition for substrates and limiting resources; 2) competitive exclusion through competition for adhesion sites; 3) synthesis of antimicrobial substances (organic acids, hydrogen peroxide and bacteriocins); 4) inhibition of toxin expression in pathogens. Each group would be explained in detail and discussed in the present chapter. Since all probiotic properties are strain-specific, including the antimicrobial activity against pathogens, there is a necessity for mandatory examination of each probiotic property of every new candidate probiotic strain. Thus, a number of methods of examination of the probiotic properties of potentially probiotic strains are being employed and developed, each method being characterized by specific techniques of examination and unravelling different pieces of information about the specific mechanisms of interaction between probiotic and pathogenic microorganisms. The existing methods for determination of the antimicrobial activity belong to two major groups: in vivo and in vitro methods. The group of in vitro methods for determination of the antimicrobial activity includes the spot-on lawn assay and its variants (simple spot-on lawn assay; agar spot assay; spot-on lawn assay with wells); the agar well-diffusion assay and disc diffusion assay and the method of co-culturing. The technique of examination of each of the enlisted methods would be described, explained and the information acquired would be analyzed in the present chapter. The in vivo methods include examinations in animal models and human clinical trials. Probiotic microorganisms are also an open field for the development of recombinant probiotics with antimicrobial peptides which offer a promising strategy against pathogens with emerging resistance against the currently used antibiotics and probiotics.

Keywords: probiotic; Lactobacillus; antimicrobial activity; pathogen; spot-on lawn assay; co-culturing

1. Probiotics – definition and health benefits

According to FAO/WHO probiotics are “live microorganisms which when administered in adequate amounts confer a health benefit to the host”. The administration of probiotics results in the prevention of a number of physical conditions and diseases and the prophylaxis and treatment of others. A number of health benefits have been observed relatively consistently in clinical studies of intestinal diseases, including:

- prevention of antibiotic associated diarrhea;
- Traveler’s diarrhea;
- reduction of duration of active diarrhea;
- reduction of respiratory tract infection incidence;
- increased eradication rates of anti-Helicobacter pylori therapy;
- prevention of severe necrotizing enterocolitis;
- symptom alleviation in irritable bowel syndrome;
- prevention of Clostridium difficile-associated diseases;
- extension or induction of remission periods in Irritable Bowel Disease patients;
- prevention of pouchitis after ileal pouch anal anastomosis;
- relieve of childhood constipation;
- prevention of atopic dermatitis in infants [2].
2. Requirements for probiotic strains

Lactobacilli and bifidobacteria are normal components of the healthy human intestinal microflora. They are included in the composition of probiotics and probiotic foods because of their proven health benefits [3]. They are the main organisms that maintain the balance of the gastrointestinal microflora.

Not all strains of lactobacilli and bifidobacteria can be used as components of probiotics and probiotic foods, but only those that meet the requirements for probiotic strains [3]:

- To be part of the natural microflora in humans and animals;
- To have the ability to adhere to epithelial cells or cell lines, or at least to be able to colonize the ileum temporarily;
- To survive in the conditions of the stomach and intestines, i.e. to survive in the conditions of acidic pH in the stomach and to withstand the effects of bile;
- To have the ability to reproduce in the gastrointestinal tract. By primarily utilizing the substrate to oppress and expel from the biological niche the pathogenic and toxigenic microorganisms;
- To possess antimicrobial activity against conditionally pathogenic, carcinogenic and pathogenic microorganisms, which is associated with inactivation of their enzyme systems, overcoming their adhesion, growth suppression and forcing them out of their biological niche, as a result of which gastrointestinal microflora is normalized;
- To produce antimicrobial substances;
- To modulate the immune response;
- To be nonpathogenic, nontoxigenic and noninvasive so that they can be safe for clinical and food applications;
- To be resistant to most of the antibiotics commonly applied in medical practice in order to be applied simultaneously with the corresponding antibiotic to preserve the balance of the gastrointestinal flora; but to be devoid of transmissible antibiotic resistance genes;
- To influence positively the metabolic activity of the consumer;
- To allow the conduction of and retain viability during and after industrial processes: industrial cultivation, resulting in obtaining concentrates with high concentrations of viable cells that can be included in gel matrices (encapsulation), thus retaining their activity in the process of freeze-drying as well as during storage of the finished products.

3. Antimicrobial activity of probiotic strains – mechanisms of interaction between probiotic and pathogenic or carcinogenic microorganisms

One of the main requirements for probiotic strains is to exhibit antimicrobial activity against pathogenic, carcinogenic and conditionally pathogenic microorganisms. The antimicrobial activity of probiotic microorganisms is strain-specific, but the mechanisms of interaction of probiotic and pathogenic microorganisms can be grouped into two groups: direct and indirect mechanisms. The direct mechanisms of interaction can be summarized in the following groups: 1) competitive exclusion through competition for adhesion sites; 2) competition for substrates and limiting resources; 3) synthesis of antimicrobial substances (organic acids, hydrogen peroxide, bacteriocins, etc.); 4) inhibition of toxin expression in pathogens.

3.1. Competitive exclusion through competition for adhesion sites

Probiotics protect the host from pathogenic and carcinogenic microorganisms by competitive exclusion, thereby hindering pathogen adhesion on the intestinal surface and the subsequent infection. Although the intestinal microflora composition is rather stable in healthy individuals and manages to withstand pathogen colonization, pathogenic and carcinogenic microorganisms become impaired when intestinal microflora is disturbed by endogenous and exogenous stress factors [4]. Adherence factors normally present on the probiotic cell surface, mostly proteins or polysaccharides, may promote pathogen exclusion, mucosal integrity and host immunomodulation. Comparative genome analyses proved the role of mucus-binding proteins (Mub) in intestinal mucus adherence of Lactobacillus strains of intestinal origin [4]. Cell surface structures such as teichoic acids, lipoteichoic acids and surface layer proteins (S-layers) are considered important for probiotic adhesion and the subsequent immunomodulation. S-layer proteins from different strains of L. acidophilus, L. helveticus, L. brevis, L. kefir and L. crispatus have been demonstrated to be involved in mediating adhesion to different host surfaces [4]. Additionally, some of them prevent adhesion of some foodborne pathogens, such as Salmonella enterica serovar Typhimurium and Escherichia coli, to frozen sections of intestinal tissue, to cultured intestinal epithelial cell lines as well as to intestinal mucus and uroepithelial cells. The anti-adhesive effect results from competition between probiotic and pathogen for the same receptor or the induction of (increased) mucin production by probiotics [5].

Several other anti-adhesiveness modes expressed by probiotics have been suggested as well: degradation of carbohydrate receptors by secreted probiotic proteins, establishing a biofilm, production of pathogen receptor analogues by probiotic cells, induction of biosurfactants and promotion of mucin production [5].
3.2. Competition for substrates and limiting resources
Probiotics and pathogenic or carcinogenic microorganisms compete for nutrients present in limited quantities in the intestine.

3.3. Synthesis of antimicrobial substances (organic acids, hydrogen peroxide, bacteriocins, etc.)
Probiotics inhibit pathogens by acidification of the gut microenvironment through production and accumulation of organic acids or by secretion of antimicrobial compounds [2].

Colonization resistance is observed in the luminal contents and the mucosal surfaces of the gastrointestinal tract. In the luminal contents, the most important resistance mechanism is the production of antagonistic metabolites by probiotic or autotchonous beneficial bacteria that suppress pathogen multiplication [4].

**Lactic acid.** One of the main metabolites produced by all lactic acid bacteria is lactic acid. Acetic acid is produced only by heterofermentative lactic acid bacteria. The accumulation of lactic acid and acetic acid and the subsequent pH reduction results in a broad-spectrum inhibitory activity against Gram-positive and Gram-negative bacteria. The acidic pH, the dissociation constant (pK value), and the mole concentration are factors that determine the inhibitory activity of lactic acid and acetic acid in the milieu. Acetic acid has more antimicrobial activity than lactic acid. Lipophilic acids such as lactic acid and acetic acid in undissociated form can penetrate the microbial cell membrane, and at higher intracellular pH, dissociate to produce hydrogen ions that interfere with essential metabolic functions of the pathogen cells such as substrate translocation and oxidative phosphorylation [6]. The toxic effects of lactic and acetic acid include intracellular pH reduction and membrane potential dissipation [4].

**Hydrogen peroxide (H₂O₂).** All lactic acid bacteria also produce hydrogen peroxide (H₂O₂) through electron transport via flavin enzymes. In the presence of H₂O₂, superoxide anions form destructive hydroxy radicals, thus, causing peroxidation of membrane lipids and increased membrane permeability. The resulting bactericidal effect of the oxygen metabolites produced is due to their strong oxidizing effect on the bacterial cell as well as destruction of nucleic acids and cell proteins [6]. H₂O₂ exhibits antimicrobial activity against yeasts, Gram-positive and Gram-negative bacteria [4].

**Bacteriocins.** Normally, the gastrointestinal tract contains many antimicrobial proteins such as colicins, defensins, cecropins, and magainins. These are low molecular weight, cationic, amphiphilic molecules, tend to aggregate and are benign to the producing organism. Lactic acid bacteria also produce a wide range of similar antagonistic factors that include metabolic products, antibiotic-like substances and bactericidal proteins, collectively termed as bacteriocins [6].

Lactic acid bacteriocins are small antimicrobial peptides or proteins that exhibit antimicrobial activity against closely related Gram-positive bacteria, whereas producer cells are immune to their own bacteriocins [4]. Even though antimicrobial peptides have a spectrum of activity narrower than conventional antibiotics, lactic acid bacteria bacteriocins can penetrate the outer membrane of Gram-negative bacteria and in combination with other augmenting antimicrobial environmental factors, such as low temperature, organic acids and detergents induce the inactivation of Gram-negative bacteria [7]. For example, lacticins A164 and BH5 are reported to be active against the Gram-negative bacterium, *H. pylori*, and nisin to be active against the Gram-negative bacterium, *H. pylori*, as well as against *L. monocytogenes* [8].

Lactic acid bacteria bacteriocins vary in spectrum of activity, mode of action, genetic origin, molecular weight, and biochemical properties. The most common bacteriocin classifications divide bacteriocins into 4 classes: (Class I) lantibiotics or small, heat-stable, lanthionine-containing, single- and two-peptide bacteriocins. The biologically inactive prepeptides of this bacteriocin class are subjected to extensive post-translational modification; (Class II) small, heat-stable, non-lanthionine-containing bacteriocins. This class is divided into 3 subclasses – Class Ila, including pediocins like or Listeria-active bacteriocins, Class Iib, containing two-peptide bacteriocins and Class Iic containing circular bacteriocins; (Class III) bacteriolysins or large, heat-labile, lytic proteins, often murein hydrolases, and (Class IV) bacteriocins that require non-proteinaceous moieties (lipid, carbohydrate) for their activity [4]. Bacteriocins can be produced spontaneously or as a result of induction. The genetic determinants of most bacteriocins are located on plasmids, with a few exceptions, which are chromosomally encoded. These antimicrobial agents are species specific and exert their lethal activity through adsorption to specific receptors located on the external surface of sensitive bacteria, followed by metabolic, biological and morphological changes resulting in the killing of sensitive bacteria [6].

Bacteriocin production is growth associated, dependent on a number of factors like pH, temperature and the presence of different enzymes. One of the major factors influencing the bacteriocin antimicrobial activity is the pH. A number of authors have found that with decreasing the pH value, the bacteriocin antimicrobial activity increases sharply. This effect of pH, for example, on bacteriocin containing no lanthionine is explained in the following way: at low pH values, the number of aggregated bacteriocin molecules is reduced and the number of free molecules that can attack the cell increases. In addition, hydrophobic bacteriocins are promoted and facilitated in their passage through the hydrophobic sections of the cell wall. Also, at elevated pH, binding of non-lanthionine-containing bacteriocins to the receptor sites of the cytoplasmic membrane can be prevented [9]. Bacteriocins isolated from *L. plantarum*, *L. brevis*, *L. casei*, *L. fermentum* and *L. jenseni* have high antimicrobial activity against *Staphylococcus aureus* and *E.coli* in the pH range of 2 to 8, with the highest activity being observed at pH=2. At pH=10-12, all bacteriocins completely lose their antimicrobial activity against the pathogens tested [10]. Another factor influencing the bacteriocin antimicrobial activity is the presence of enzymes such as proteinase K, trypsin and other proteases, lipase, peroxidase, α-amylase. In the
presence of proteinase K and trypsin, bacteriocins synthesized by *L. reuteri* 2-20B and *Pediococcus acidilactici* 0-11A lose their antimicrobial activity [11]. Temperature is also a factor affecting the bacteriocin antimicrobial activity.

Bacteriocins synthesized from *L. reuteri* 2-20B and *Pediococcus acidilactici* 0-11A retain their antimicrobial activity after heat treatment at 80 °C for 1 hour. Bacteriocins produced by *L. reuteri* 2-20B lose their activity at a temperature of 121°C for 15 minutes and at 100°C for 1 hour [11].

Many modes of action have been described for lactic acid bacteria bacteriocins. The primary target for many bacteriocins is the cytoplasmic membrane of sensitive bacteria. The dissipation of the proton motive force induces the formation of discrete pores, resulting in energy loss. Other bacteriocins do not form membrane pores, but interfere with essential enzyme activities in susceptible bacteria. Most bacteriocins interact with anionic lipids in the membranes, and consequently initiate pore formation in the membranes of susceptible cells [4]. Some lactic acid bacteria species produce only one bacteriocin, while others can synthesize multiple bacteriocins (2-3 bacteriocins) [6].

Lactic acid bacteria bacteriocins possess a number of desirable properties that make them suitable for food preservation: (a) they are GRAS-substances (Generally Recognized As Safe), (b) they are not active and nontoxic on eukaryotic cells, (c) they are readily inactivated by digestive proteases, with slight impact on the gut microorganisms, (d) they can tolerate a wide range of pH and temperature, (e) they have a fairly broad antimicrobial spectrum, against many food-borne pathogens and food spoilage bacteria, (f) they exhibit bactericidal action, typically acting on the bacterial cytoplasmic membrane: no cross resistance with antibiotics, and (g) their genetic determinants are usually plasmid-encoded, facilitating genetic manipulation [7].

**Carbon dioxide.** Carbon dioxide is produced by heterofermentative lactic acid bacteria and exhibits antimicrobial activity against most taxonomic groups of microorganisms. It is a major end product of hexose fermentation by heterofermentative lactic acid bacteria. A number of lactic acid bacteria are capable of CO₂ production from malate and citrate. They can also metabolize arginine via the arginine deaminase pathway to form CO₂. Finally, decarboxylation of amino acids (histidine, tyrosine) can also result in CO₂ formation [6]. CO₂ creates anaerobic environment by replacing existing molecular oxygen. It can decrease pH and has destructive effects on cell membranes [6].

**Diacetyl.** Diacetyl, acetaldehyde and acetoin are produced by a variety of lactic acid bacteria and are also active against yeasts, Gram-positive and Gram-negative bacteria.

Diacetyl (2,3-butanediene) is an end product of pyruvate metabolism by citrate fermenting lactic acid bacteria. It exhibits antimicrobial activity against various food-borne pathogens and spoilage microorganisms. Diacetyl is more effective against Gram-negative bacteria, yeasts and molds, than against Gram-positive microorganisms. Diacetyl reacts with the arginine-binding protein of Gram-negative bacteria, hence, interfering with arginine utilization. Concentrations of 0.2 mg/ml and 0.3 mg/ml are required for exhibiting antimicrobial activity against yeasts / Gram-negative bacteria, and non-lactic Gram-positive bacteria, respectively [6].

**Acetaldehyde** possesses antimicrobial activity (10-100 ppm) against food-borne pathogens, *E.coli*, *Salmonella typhimurium* and *S.aureus* [6].

**Other low molecular mass antimicrobials.** There are other low molecular mass compounds with antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi and yeasts produced by lactic acid bacteria such as antifungal cyclic dipeptides, phenyllactic acid, 4-hydroxyphenyllactic acid and 3-hydroxy fatty acids [4]. Cyclic dipeptides are produced by *Lactobacillus plantarum* and *Lactobacillus pentosus*. 3-phenyllactic acid and 4-hydroxyphenyllactic acid are formed by many lactic acid bacteria (*Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus sanfranciscensis*, *Lactobacillus brevis*, *Lactobacillus acidophilus*, etc.), while 3-hydroxy fatty acids are produced by *Lactobacillus plantarum*. Cyclic dipeptides, 3-phenyllactic acid, 4-hydroxyphenyllactic acid and 3-hydroxy fatty acids exhibit antifungal activity. *Lactobacillus plantarum* produces benzoic acid, methylhydantoin and mevalonolactone which inhibit the growth of fungi and Gram-negative bacteria [4].

**Deconjugated bile acids.** Deconjugated bile acids have a stronger antimicrobial activity against Gram-positive and Gram-negative pathogens compared to the bile salts synthesized by the host organism [5].

**Cell surface polysaccharides.** Polysaccharides are ubiquitously present in *Lactobacillus* cell walls. The cell surface polysaccharide structures vary considerably in terms of sugar compositions, sugar-linkages, polymer branching, as well as their specific modifications such as phosphorylations, acetylations, and pyruvylations. Most lactobacilli surface polysaccharides are heteropolysaccharides, in which the polymer is composed of regular repeating units that contain different sugars, commonly including D-glucose, D-galactose and L-rhamnose, and in a few cases also N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc) or glucuronic acid (GlcA) [2]. The biological functions of the lactobacilli cell surface polysaccharides are related to phage absorption (*L. plantarum* and *L. casei*), the attachment of surface layer proteins (*Lactobacillus buchneri*), and immunomodulation [2]. Exopolysaccharides contribute to the adhesion of lactic acid bacteria to the intestinal mucosa and protect the cells from the low acidity in the stomach. As a result, the survival of lactobacilli passing through the stomach increases, resulting in an increase in the concentration of active cells in the gastrointestinal tract [12]. Exopolysaccharides exhibit antimicrobial activity against *Escherichia coli*, *Listeria monocytogenes*, Staphylococcus aureus, Candida albicans, Bacillus cereus, Salmonella typhimurium, Shigella sonnei, Shigella flexneri CMCC, Escherichia coli 0157, Pseudomonas aeruginosa [13, 14]. A number of lactobacilli exopolysaccharides also have a pronounced antioxidiant effect, anticancer action, and lead to a reduction in bad cholesterol [13]. Exopolysaccharides protect the producing cells from protein denaturation during
drying. They also protect the cells from osmotic shock, freezing, some antibiotics, toxic chemicals and a number of physical factors. They also play a major role in cell adaptation to various extreme conditions [12]. Numerous exopolysaccharides have been found to stimulate the growth of bifidobacteria and lactic acid bacteria in the gastrointestinal tract and lead to an increase in the concentration of beneficial microflora in the intestinal canal [14, 15].

3.4. Inhibition of toxin expression in pathogens

Toxins are the most important group of bacterial virulence factors. The effectiveness of certain probiotics in diarrhoea is based on their ability to protect the host against toxins. The observed protection can be the result of inhibition of toxin expression in pathogens [5].

4. Methods of examination of the antimicrobial activity of probiotics against pathogens

Although some health benefits may be less dependent on the probiotic strain used, many of the beneficial effects of probiotics, including the antimicrobial activity against pathogens, are strain specific, implying that the proposed efficacy of a particular strain cannot be extrapolated to other probiotic strains or species [2]. Thus, there is a necessity for mandatory examination of each probiotic property of every new candidate probiotic strain. A number of methods of examination of the probiotic properties of potentially probiotic strains are being employed and developed, each method being characterized by specific techniques of examination and unravelling different pieces of information about the specific mechanisms of interaction between probiotic and pathogenic microorganisms. The existing methods for determination of the antimicrobial activity belong to two major groups: in vivo and in vitro methods. The group of in vitro methods for determination of the antimicrobial activity includes the spot-on lawn assay and its variants (simple spot-on lawn assay; agar spot assay; spot-on lawn assay with wells); the agar well-diffusion assay and disc diffusion assay and the method of co-culturing. The in vivo methods include examinations in animal models and human clinical trials.

4.1. In vitro methods for determining the antimicrobial activity of probiotics against pathogens

4.1.1. Spot-on lawn antimicrobial assays

**Simple spot-on lawn antimicrobial assay (Fig. 1).** The method has the following steps: 1) Different nutrients, selective or differential media, are prepared and the chosen pathogen at different initial concentrations is either spread plated after hardening of the agar medium or is mixed with the melted and cooled agar medium prior to pouring into the Petri dishes. 2) Different dilutions of the investigated probiotic microorganism or acellular supernatant (containing bacteriocins and organic acids) or neutralized acellular supernatant (the organic acids are neutralized) are then spotted onto the medium already inoculated with the chosen pathogenic microorganisms [16]. 3) After incubation, the antimicrobial activity is expressed either as inhibition zone or as arbitrary units (AU/mL). The inhibition zone is recorded either as the diameter or the area of the inhibition zone. Arbitrary units are the reciprocal of the highest dilution at which the growth of the pathogen is inhibited and are calculated as \((1000/a) \times D\) in AU/mL, where \(a\) is the volume (μL) of the spotted sample and \(D\) is the dilution factor [16].
Agar spot-on-lawn antimicrobial assay (Fig. 2). The method consists of the following steps: 1) Selective or differential media are prepared and poured into Petri dishes. 2) Different dilutions of the investigated probiotic microorganism or acellular supernatant (containing bacteriocins and organic acids) or neutralized acellular supernatant (the organic acids are neutralized) are then spotted onto the medium. The Petri dishes are then incubated to develop spots. 3) A suspension of the pathogenic microorganism is mixed with specific soft agar (0.7%) and poured over the spotted Petri dishes. 4) The Petri dishes are 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 [16].
**Spot-on lawn antimicrobial assay with wells (Fig. 3).** The method is characterized by the following steps: 1) Selective or differential media are prepared and poured into Petri dishes. 2) Wells (6 mm in diameter) are prepared in each Petri dish and the bottom of the wells is sealed with agar. 3) Different dilutions of the investigated probiotic microorganism or acellular supernatant (containing bacteriocins and organic acids) or neutralized acellular supernatant (the organic acids are neutralized) are pipetted into the wells. The Petri dishes are left at room temperature to allow migration and settling of the test samples. 4) After that the samples are incubated for 3 h at 37°C and the Petri dishes are then overlaid with agar inoculated with the pathogenic microorganism and incubated at suitable incubation conditions. After incubation, the antimicrobial activity is recorded either as inhibition zone or as arbitrary units (AU/mL) [16]. This method is simple and effective for screening the potential antimicrobial activity of lactic acid bacteria. However, there is a problem with this technique - the possible contamination that occurs during the overlay stage, complicating the interpretation of inhibition zones. Many modifications aiming at avoiding this pitfall have been suggested, such as the use of methanol vapours to kill lactic acid bacteria prior to the overlay step, or the reverse side agar technique based on the tridimensional diffusion of bacteriocins. Another solution is a technique combining the spot-on lawn and well diffusion methods, whereby *Lactobacillus* containing sample is pipetted in a well that is filled with agar before the overlay step, thus permitting reduced contamination risk [17]. This method modification avoids the masking of inhibition zones and ensures the clear and reliable detection of the antimicrobial activity of *Lactobacillus* strains [17].

**Cross streak antimicrobial assay (Fig. 4).** The method technique is the following: each probiotic strain is streaked in three parallel lines onto suitable agar medium using a loop. The lines are left to dry and then the test pathogenic strain is streaked perpendicular to these initial strains in the same fashion. When there is inhibition, it is caused by the tested probiotic strain hindering the growth of the second streaked (pathogenic) microorganism [16].

**Fig. 3** Spot-on-lawn antimicrobial assay with wells.
4.1.2. Agar well-diffusion antimicrobial assay and paper disc antimicrobial assay

**Agar well-diffusion antimicrobial assay (Fig. 5).** This method is commonly used for determining the antagonistic effects of acellular supernatants. The assay consists of the following steps: 1) different nutrients, selective or differential media, are prepared. The Petri dishes are inoculated (spread-plating or pour-plating) with the chosen pathogenic microorganism. 2) 6-mm or 7-mm wells are prepared in each Petri dish. 3) Aliquots of different dilutions of acellular supernatants are pipetted into the wells. After incubation, the antimicrobial activity is recorded either as inhibition zone or as arbitrary units (AU/mL) [16].
**Paper disc antimicrobial assay (Fig. 6).** The method includes the following steps: 1) different nutrients, selective or differential media, are prepared. The Petri dishes are inoculated (spread-plating or pour-plating) with the chosen pathogenic microorganism. 2) paper discs (6 mm in diameter) are soaked and absorb aliquots of acellular supernatant and are then placed on the hardened agar medium. After incubation, the inhibition zone is evaluated based on the clear zone around the paper disc [16].

![Paper disc antimicrobial assay](image)

**Fig. 6** Paper disc antimicrobial assay.

4.1.3. Co-culturing assays

**Simple co-culturing assay.** This method includes the following steps: 1) preparation of incubation media; 2) aliquots of a pathogenic and a probiotic microorganism are inoculated into the incubation media. The samples are mixed well and incubated. 3) After incubation, the number of viable cells of the pathogenic and probiotic microorganisms are determined on appropriate agar medium. Values are usually expressed as log cfu/ml [16].

**Microtitre plate assay.** It includes the following steps: 1) acellular supernatant of active probiotic microorganism that is divided into several parts that undergo different treatments (neutralization with NaOH added; heat treatment, etc.) is prepared. 2) the chosen pathogenic microorganism is cultured and added to appropriate broth with the acellular supernatant. 3) Incubation at suitable incubation conditions. Before and after incubation, the optical density at 620 nm is measured and the inhibitory activity of the acellular supernatant is calculated as a percentage of inhibition of pathogen growth [16].

**Co-culturing assay using cell lines.** The evaluation of probiotic effects on human intestinal cell lines in vitro is meaningful as the cell line cells simulate the gut microenvironment. Thus, they are used as a biological matrix alternative to in vivo tests. In fact, in vitro experiments and research are particularly important since the EU directives actually discourage in vivo experiments on animals. Several different cell lines have been used for the examination of the antimicrobial activity of probiotic microorganisms against pathogenic and carcinogenic microorganisms. For example, HT-29 cell line from human colon, IPEC-J2 porcine neonatal jejunal cell line, Vero African green monkey kidney epithelial cell line, Caco-2 colon adenocarcinoma cell line, HIEC-6 normal epithelial small intestine cell line, BALB/c3T3 murine embryonal fibroblast cell line and many others. Cells are grown in Dulbecco's modified Eagles' medium (DMEM), McCoy's 5a medium or other medium and inoculated in well plates or microtitre plates that are incubated at 37°C for 24 h in 1 mL medium in the presence of 5% of CO₂. Probiotic and pathogenic microorganisms are then added and the number of viable probiotic and pathogen cells is determined after different periods of incubation [16].
It is difficult to extrapolate the observed in vitro effect to the gastrointestinal environment in vivo. In fact, in vivo the resident microbiota and mucus, and the peristaltic flow that continuously washes the gastrointestinal epithelium, could efficiently modify adhesion of exogeneous Lactobacillus and Bifidobacterium bacteria [8].

4.2. In vivo methods for determining the antimicrobial activity of probiotics against pathogenic microorganisms

To ascertain that the antimicrobial activity of probiotic Lactobacillus and Bifidobacterium bacteria observed in vitro also happen in vivo, blocking the epithelium colonization or inhibiting invasiveness, appropriate infectious animal models should be used. Unfortunately, some pathogenic bacteria and viruses could not be investigated using animal models since they are highly specific for human tissues, mainly as the result of structural and functional differences in the intestinal epithelia of humans and animals [8].

4.2.1. In vivo examination using animal models

When conducting in vivo animal model antimicrobial assay all animal models include at least two groups under controlled settings. The first group receives the chosen probiotic and pathogen (treated infected group) and the second one receives only the pathogen (untreated infected group). After a specific period, there are examinations of faeces as well as the examination of different cells after scarifying the animals (spleens, lymph nodes, blood, liver, colon, cecum, etc.) to detect and analyze the differences. Animals used in these studies include mice, rats, chicks, rabbits, pigs, fish and even worms. The whole animal trial procedure is carried out in accordance with the guidelines of the European convention for the protection of vertebrate animals used for experimental and other scientific purposes [16].

While some animal models are often able to closely simulate human physiology and disease [18], they are mostly used to study the molecular effectors and the mechanisms of probiotic action. The animal models do not always provide scientifically appropriate, relevant and applicable results for human, due to obvious species-specific differences in anatomy, biochemistry, physiology, pharmacokinetics and toxic responses [16].

4.2.2. In vivo examination using human trials

The in vivo examination using human trials includes randomized double blind, placebo-controlled human trials aiming at establish the efficacy of a probiotic product. There is a need for human studies in which adequate numbers of subjects are enrolled to achieve statistical significance [16]. A number of well-designed, double-blind, placebo-controlled clinical trials have been conducted in order to demonstrate that a selected lactobacilli or bifidobacteria strain does actually display these properties in humans developing the disease(s) [8]. Presently, there is evidence for their adhesiveness properties or persistence in the gut and health benefits in diarrhoea and Helicobacter pylori infection.

5. Future perspectives

5.1. Heterologous expression of antimicrobial peptides (AMPs) in probiotic Lactobacillus strains

The constant emergence and outbreaks of infections caused by resistant pathogenic infectious bacteria is a contemporary challenge to pharmaceutical companies worldwide. Such pathogens are Enterococcus sp., Staphilococcus aureus, Klebsiella sp., Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter sp. Furthermore, some Gram-negative bacteria strains are unaffected by all the presently available antibiotics. The resistance of some bacterial strains combined with their high infectivity are also causing many problems in all parts of the world [19]. Some food-related and commensal lactic acid bacteria with GRAS-status are promising live vehicles for the delivery of proteins or peptides of medical or veterinary interest, which is a trend that is gaining momentum [18].

AMPs can be: (i) colonizing peptides, assisting the probiotic strain to compete successfully with the resident flora or invading pathogens for adhesion sites; (ii) killer peptides, resulting in pathogen elimination; or (iii) signal peptides, recruiting other resident microflora in the gut or the host immune system to fight against and eliminate the pathogens [19].

Current methods of production, purification and delivery of AMPs have several limitations: 1) Commonly, AMPs cannot be orally administered because they are rapidly degraded before reaching their target site. 2) Antimicrobial peptides are quickly identified and targeted by the host immune system before they can reach the infection site. Therefore, high AMP concentrations must be applied in order to achieve a therapeutic effect. But this strategy has high production costs, and in some cases causes enormous toxic side effects (e.g. hemolysis). Thus, the development of AMP based therapeutics is limited [19].

One way to solve these challenges is to use probiotics to produce and secrete heterologous endogenous AMPs with desired properties and effects on the host [19]. Unfortunately, heterologous expression of AMP in bacteria is still an enormous challenge because bacterial host can be killed due to high AMP concentrations [19]. Furthermore, the use of
genetically modified lactic acid bacteria is always accompanied by the risk of unwanted spread in the environment even if the modified strains are intended to be applied to well-confined niches in the human body. The permanent colonisation of the host by the engineered strain may also be undesirable. For these reasons, the biocontainment of the modified strains is of great importance [18]. When developing genetically modified lactic acid bacteria, the general idea is to use the minimum amount of foreign DNA to modify the selected strain, to avoid antibiotic resistance markers for counter selection and, even if the genetic modification is chromosomally integrated, to minimise the possibility for its horizontal gene transfer. Ultimately, any possible release of the engineered strain to an unconfined environment should be lethal [18]. A possible strategy to achieve this aim is to make the strain auxotroph for an essential metabolite. Supplementation with this essential metabolite will be necessary for the administration of the live engineered strain to the host. Release of the engineered strain to an unconfined environment should result in cell death due to scarcity of the essential metabolite [18].

5.2. Designer probiotics

Some probiotic strains are designed to express the receptor for certain pathogen toxin and they bind it very efficiently, thus, protecting the host organism. Such examples are designer probiotics that bind shigatoxin 1 (Stx1) and 2 (Stx2).

Similar designer probiotics carrying the receptor for the heat-labile enterotoxin of enterotoxigenic E. coli (ETEC) or the receptor for cholera toxin very efficiently bound the respective toxin and showed good protection in animal models after ETEC or Vibrio cholerae challenge [5].

5.3. Lactic acid bacteria as live vaccines

Probiotic cells can be engineered to express many antigens (virus, pathogen, etc.) that would stimulate the host immune system to develop resistance mechanisms in order to prevent infection by the same disease-causing agent. Strains of L. casei, L. plantarum, L. rhamnosus and L. acidophilus have been used as live vaccines. Lactobacilli have been engineered to express many antigens including the HPV-16 E7 antigen, the HIV-1 Gag protein, the adhesin Hp0410 from H. pylori, the prostate-specific antigen (PSA), etc. [18].

Bifidobacteria have been used as recombinant hosts rather infrequently, but they have found application as live vaccines in some instances expressing antigens like the VP1 protein from Enterovirus or the Hepatitis C NS3 peptide.

Sometimes the antigen producing mutant strain is co-administered with a strain modified to produce or co-express molecules with important immunomodulatory properties like leptin and IL-12 to improve immunisation. Some of the lactic acid bacteria plasmid vectors available can target protein production and be cytoplasmic, secreted to the extracellular environment or even anchored to the cell surface by including or excluding specific signal sequences because the location of the protein antigen has been shown to influence its immunogenicity. The strongest immune response on the host is triggered by antigens exposed to the surface of lactic acid bacteria anchored to the cell wall. Hence, most live lactic acid bacteria vaccines are now being designed for surface exposed antigens [18].

6. Conclusion

Studies in the field of probiotics are a neverending path of isolation, identification and examination of the probiotic properties of different strains including their antimicrobial activity against pathogenic, conditionally pathogenic and carcinogenic microorganisms. The aim of these studies is developing and engineering new and improved probiotic strains and formulations to assist the host immune system and ensure the preservation of human health.

References
