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Short Communication

Assessment of the in vitro antimicrobial activity of *Lactobacillus* species for identifying new potential antibiotics

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ABSTRACT

The bacteriocin-mediated antimicrobial properties of *Lactobacillus* spp. have been widely studied, leading to the use of these micro-organisms in the food industry as preservative agents against foodborne pathogens. In an era in which antibiotic resistance is becoming a public health issue, the antimicrobial activity of *Lactobacillus* spp. could be used for the discovery of new potential antibiotics. Thus, it is essential to have an accurate method of screening the production of antimicrobial agents by prokaryotes. Many in vitro assays have been published to date, largely concerning but not limited to *Lactobacillus* spp. However, these methods mainly use the spot-on-the-lawn method, which is prone to contamination during the overlay stage, with protocols using methanol vapours or the reverse side agar technique being applied to avoid such contamination. In this study, a method combining the spot-on-the-lawn and well diffusion methods was tested, permitting clear identification of inhibition zones from eight *Lactobacillus* spp. towards clinical isolates of 12 species (11 bacteria and 1 yeast) commonly found in human pathology. *Lactobacillus plantarum* CIP 106786 and *Lactobacillus rhamnosus* CSUR P567 exhibited the widest antimicrobial activity, whereas *Lactobacillus acidophilus* strain DSM 20079 was relatively inactive. In addition, the putative MIC_{50} of *L. rhamnosus* against *Proteus mirabilis* was estimated at 1.1×10^9 CFU/mL using culture broth dilution. In conclusion, considering the increasing cultivable bacterial human repertoire, these findings open the way of an effective method to screen in vitro for the production of potential antimicrobial compounds.

1. Introduction

Interest in probiotics has grown over the last 10 years, despite controversial opinions about their effects on human health. *Lactobacillus* spp., a constituent of probiotics when associated with *Bifidobacterium* spp., possess strong antibacterial properties through bacteriocins. Antimicrobial agents with variable spectra have been isolated and characterised from strains of *Lactobacillus acidophilus* [1,2], *Lactobacillus reuteri* [3], *Lactobacillus rhamnosus* [4] and *Lactobacillus casei* [5], organisms that are commonly used in the preparation of probiotics. In addition, secretion of bacteriocins, preferentially under anaerobiosis, has been implemented in the food industry to inhibit foodborne pathogens [6]. In an era in which new antibiotics are lacking [7] and resistance is increasing [8], the use of such micro-organisms as antibiotics should also lead to consider the application of these molecules as potential antimicrobial agents.

Several methods have been developed to screen for the antimicrobial activity of bacteria, particularly *Lactobacillus* spp. The most used and popular method remains the spot-on-the-lawn method [9], which consists of incubation of *Lactobacillus* spp., mainly on acid agar, under anaerobiosis; a soft agar seeded with the tested indicator organism is then overlaid following the formation of colonies. Following growth of the indicator, inhibition zones can be measured. This method is simple and effective for screening the potential antimicrobial activity of lactic acid bacteria (LAB). However, the problem with this technique is the possible contamination that occurs during the overlay stage, complicating the interpretation of inhibition zones. Thus, many attempts to avoid this pitfall have been described, such as the use of methanol vapours to kill LAB strains prior to the overlay step [10,11], or the reverse side agar technique based on the tridimensional diffusion of bacteriocins [12]. Here we propose a test combining the spot-on-the-lawn and well diffusion methods, whereby *Lactobacillus* strains grow in

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a hole that is filled with agar before the overlay step, thus permitting reduced contamination risk. This work is a preliminary step in the larger screening of potential new antibiotics produced from various micro-organisms.

2. Materials and methods

2.1. Lactobacillus *strains and culture conditions*

Eight strains of *Lactobacillus* were included in this study: *L. acidophilus* (DSM 20079); *Lactobacillus gasseri* (CIP 102991); *Lactobacillus fermentum* (CIP 102980); *Lactobacillus ingluviei* (CSUR P209); *Lactobacillus plantarum* (CIP 106786); *L. reuteri* (DSM 20016); *Lactobacillus sakei* (DSM 20017); and *L. rhamnosus* (CSUR P567). All *Lactobacillus* strains were incubated for 24 h on Columbia agar plates (bioMérieux, Craponne, France) under aerobic conditions with 5% CO_2.

2.2. *Indicator strains*

The indicator strains were clinical isolates selected according to their antibiotic resistance pattern. Wild-type strains were selected for *Escherichia coli*, *Enterococcus faecalis*, *Enterococcus faecium*, *Enterobacter cloacae*, *Streptococcus salivarius*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Bacteroides thetaiotaomicron*. A clinical isolate each of *Candida albicans* and *Clostridium difficile* DSM 12057 were also chosen as indicators. All strains were incubated for 24 h on Columbia agar plates in the appropriate atmosphere before testing.

2.3. *In vitro screening assay*

First, a pre-culture step of lactobacilli was performed to assess their capacity to produce antimicrobial components. In Petri dishes, eight small holes (ca. 6 mm in diameter) were punched in MRS agar (Sigma-Aldrich, Saint-Quentin-Fallavier, France) and droplets of MRS agar were added to each hole to line the bottom of the Petri dishes. Then, 5 μL of a *Lactobacillus* strain suspension containing ca. 10^8 cells/mL in tryptic soy broth (TSB) (BD Diagnostics, Le Pont-de-Claix, France) was inoculated into each hole and was incubated for 12–24 h at 30 °C under anaerobic or aerobic conditions. For the preparation of indicators previously grown on Columbia blood agar (bioMérieux, Marnes-la-Coquette, France), the basic medium was brain–heart infusion (BHI) (BD Diagnostics) added to the agar to obtain a concentration of 5 g/L. Some colonies of the target bacteria were mixed in 17 mL of BHI broth to obtain an approximate concentration of 10^7 cells/mL; the mixture was then overlaid onto the surface of the MRS agar containing the *Lactobacillus* colonies. The plates were incubated under the appropriate atmosphere, depending on the indicator strain, for 24–48 h at 37 °C.

The same procedure, with a few modifications, was applied for testing *Lactobacillus* activity against anaerobes. In this modified version, the *Lactobacillus* pre-culture step was performed on MRS agar under anaerobic conditions for 24 h; a few colonies were then suspended in TSB and were inoculated in holes previously punched in Wilkins–Chalgren agar (BD Diagnostics). The indicator strains were mixed in 17 mL of Wilkins–Chalgren soft agar (5 g/L) to obtain an approximate concentration of 10^7 cells/mL and then the mixture was overlaid onto the surface of the Petri dishes containing the lactobacilli. The cultures were incubated in an anaerobic cabinet for 24 h at 37 °C.

Finally, the diameters of growth inhibition of the tested bacteria were measured to determine the antimicrobial activity of the *Lactobacillus* spp., and the Petri dish was photographed using a Scan 1200 (Interscience, St-Nom-la-Bretêche, France). An inhibition zone with a diameter >5 mm was recorded as positive (Table 1; Fig. 1).

2.4. *Determination of the putative minimum inhibitory concentration (MIC)*

Determination of the putative MIC was performed using *L. rhamnosus* as the inhibitory strain and *P. mirabilis* as the indicator strain. Briefly, *L. rhamnosus* was inoculated in MRS broth and was grown for 12 h under anaerobic conditions; *P. mirabilis* was grown in BHI broth under aerobic conditions. The bacterial load was then estimated using the plate count agar method; 500 μL of each culture was added to BHI broth supplemented with linezolid (20 mg/L) (Pfizer, Paris, France) to stop *Lactobacillus* growth (broth A) and was incubated for 24 h. Two negative controls consisting of *P. mirabilis* alone (broth B) and *P. mirabilis* added to MRS broth and linezolid (20 mg/L) (broth C) were used. Viable cells were counted on MRS agar and MacConkey agar (BD Diagnostics) for *L. rhamnosus* and *P. mirabilis*, respectively.

3. Results

3.1. *Methods*

To compare antimicrobial activity under aerobic and anaerobic conditions, *Lactobacillus* incubation on MRS agar was performed under both atmospheres. Anaerobic pre-incubation allowed for a larger inhibition zone and the absence of aspecific inhibition, justifying the incubation of *Lactobacillus* under anaerobic conditions for screening their antimicrobial activity.

Contamination was observed with the classic spot-on-the-lawn method. Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) applied to these colonies identified *Lactobacillus* spp., suggesting that the contamination occurred during the overlay stage. The well diffusion method combined with the spot-on-the-lawn protocol permitted a reduction in contamination risk and the visualisation of clear inhibition zones (Fig. 1). Finally, the use of Wilkins–Chalgren agar for testing anaerobes was necessary, as shown by the absence of indicator growth when MRS agar and BHI were used (data not shown).

3.2. *Antimicrobial activity of* Lactobacillus *spp. included in the study using the spot-on-the-lawn method*

Among the eight *Lactobacillus* strains tested, *L. plantarum* CIP 106786 and *L. rhamnosus* CSUR P567 showed the best antimicrobial activity (11/12 indicators inhibited), followed by *L. reuteri* DSM 20016 (8/12 indicators inhibited), whereas *L. acidophilus* DSM 20079 was the least active (2/12 indicators inhibited). Among the indicators tested, *C. albicans* was not inhibited by any *Lactobacillus* strain. The results are summarised in Table 1.

3.3. *Putative minimum inhibitory concentration of* Lactobacillus rhamnosus *against* Proteus mirabilis

The final counts of *P. mirabilis* showed a four-fold decrease between broth A with *L. rhamnosus* and control broths B and C (Fig. 2). No difference was observed between broths B and C. The initial *L. rhamnosus* concentration was estimated at 2.2×10^9 CFU/mL, and its MIC_{50} (MIC that inhibits 50% of the bacterial isolates) towards *P. mirabilis* was evaluated as 1.1×10^9 CFU/mL.