BJPS

Brazilian Journal of
Pharmaceutical Sciences

http://dx.doi.org/10.1590/s2175-97902018000117533

Short Communication

Importance of the agar-media in the evaluation of bacteriocin activity against the same test-microorganisms

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Bacteriocins are peptides produced by various species of bacteria, especially lactic acid bacteria, which exhibit a large spectrum of action against spoilage bacteria and foodborne pathogens. Successful application of techniques for quantitative or qualitative bacteriocin determination relies not only on the sensitivity of the test-microorganisms, but also on the agar-medium employed. Cell free supernatants are routinely used to preliminary screen for antimicrobial activity of bacteria by means of the agar well diffusion method, but the supernatant may also include other molecules (such as medium components and/or intracellular compounds) accidentally released during cell free supernatant preparation, which may interfere with the assay. Reproducibility of bacteriocin activity against the same test-microorganisms is an important factor to be considered. Unfortunately, no specific information about bioassays standardization to determine bacteriocin activity is available in the literature. In this work, growth inhibition by means of the agar well diffusion assays were carried out on different agar-media showing a strong dependence on the agar-medium used, indicating that the inhibitory effects could also depend on the diffusion of exudates that are included in the cell-free supernatant. The results presented in this communication show that selection of the agar-medium is crucial for the bioassay response.

Keywords: Bacteriocin. Growth inhibition. Solid medium. Agar well diffusion assay

INTRODUCTION

Bacteriocins are small and cationic antimicrobial peptides, ribosomally synthesized by lactic acid bacteria (LAB) (Singh, Ghosh, 2012), with antilisterial activity (Papagianni, Sergelidis, 2015) and many industrial applications (Papagianni, 2003). The industrial importance of LAB is further demonstrated by their status generally recognized as safe (GRAS) (Fan, Song, 2013). Additionally, lactic acid and other metabolic products produced by LAB contribute to the organoleptic and textural profile, as well as shelf life of food products (Ross, Morgan, Hill, 2002), especially the ones produced from fermented milk cultures concerning the control of foodborne pathogens (Porto *et al*., 2017).

Several pediocins, the bacteriocins produced by *Pediococcus pentosaceus* spp., have been characterized (Papagianni, Anastasiadou, 2009) and they are amongst the most promising bacteriocin in the industry (Turcotte *et al*., 2004) as food preservative (Bharti *et al*., 2015). They have structural similarities, but different spectrum of antimicrobial activity (Papagianni, Sergelidis, 2015). Pediocins exhibit important technological properties such as thermostability and the capacity of retaining activity at a wide range of pH with bactericidal action especially against Gram-positive food spoilage and pathogenic bacteria; these features make pediocins an important class of biopreservatives (Papagianni, Anastasiadou, 2009).

P. pentosaceus ATCC 43200, also known as FBB61, was isolated in 1953 from cucumber fermentation and produced pediocin A (Fleming, Etchells, Costilow, 1975), which was shown to belong to the class III bacteriocins (Klaenhammer, 1993), with molecular weight of 80 kDa and a broad range of activity against Gram-positive bacteria (Piva, Headon, 1994).

A major difficulty in antimicrobial peptide research and application is their identification and quantification using bioassays (Choyam *et al*., 2015). Agar diffusion

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assay, that produces halos, where growth is inhibited, is undoubtedly the most commonly used method to determine bacteriocin activity (Bouksaim *et al*., 2000). However, this methodology is dependent on the bacteriocin diffusing through the agar, which is time-consuming and also relies on human interpretation when zones of inhibition are unclear or not perfectly circular (Papagianni *et al*., 2006). The possibility of unspecific reaction between the active substance present in the tested culture and the agar-medium should also be considered.

The purpose of this work was to illustrate seemingly just how variable zones of inhibition can be when bacteriocin activity against *Enterococcus* spp., *Listeria* spp. and *Escherichia coli* was assessed using agar diffusion test performed in different types of agar-media.

MATERIAL AND METHODS

Bacterial strains and growth conditions

P. pentosaceus ATCC 43200 (OD_{600nm} = 0.8-0.9) (Halo VIS-10, Dynamica, Australian) was grown at 30 °C, stirring at 100 rpm (TE-420, Tecnal, Piracicaba, SP, Brazil), in 100 mL of pH 6.5 Man, Rogosa and Sharpe (MRS) medium (Difco Laboratories, Detroit, MI, USA), which contains 2.0% (w/v) as main C-source (G-MRS). It was supplemented with either 1.0% (w/v) inulin (Orafti®GR, Orafti Active Food Ingredients, Oreye, Belgium) (GI-MRS), 1.0 % (w/v) sucrose (GS-MRS), 1.0 % (w/v) inulin and 1.0% (w/v) sucrose (GSI-MRS); alternatively, 2.0% (w/v) inulin (I-MRS) and 2.0% (w/v) sucrose (S-MRS) were used as the sole carbon source. Aliquots were taken every 2 h during the first 12 h of the cultivation, and thereafter at 24 h and 48 h.

Enterococcus spp. (strains Ent101, Ent104 and Ent711), *Listeria innocua* (strains Li2052 and Li2865) were grown in Brain Heart Infusion (BHI) medium (Difco). *Escherichia coli* ATCC 25922 was grown in Tryptic Soy (TSB) Broth (Difco). One mL of each cryopreserved bacterium was grown in 5.0 mL medium for 16 h at 37 °C without stirring (TE-310, Tecnal, Piracicaba, SP, Brazil). The cultures were then diluted with sterile deionized water to provide an optical density of 600 nm for 0.3.

Bacteriocin activity determination

P. pentosaceus cells were collected by centrifugation (4.470 x *g* at 4 °C for 15 min) and the cell-free supernatant was adjusted to pH 6.0-6.5 by the addition of 1.0 N NaOH, heated to 70 °C for 25 min to inactivate proteases and filtered (0.45 µm pore diameter filter, Millipore, Billerica, MA, USA). The agar well diffusion assay was performed using 200 µL of each indicator strain suspension (OD = 0.3) (Halo VIS-10, Dynamica, Australian) added to 20.0 mL of medium supplemented with 1.0 % (w/v) agar and poured into Petri dishes. Fifty µL of each denatured supernatant was added into each well. All plates were incubated for 16-18 h at 37 °C (TE-310, Tecnal, Piracicaba, SP, Brazil) in duplicate. The agar-media evaluated were BHI and Mueller Hinton to *Enterococcus* and *Listeria* strains and TSB and MacConkey to *E. coli*.

Statistical analysis

One-way analysis of variance (ANOVA) and Tukey's test by Microsoft Excel (Redmond, WA, USA) were performed and a significant difference was defined as a p value < 0.05.

RESULTS AND DISCUSSION

The production of bacteriocin is dependent on the type of nutrient sources present in the medium (Todorov, Dicks, 2004) and to confirm the unsuitability of sucrose and inulin as the only carbon source for this *P. pentosaceus* strain, as demonstrated by de Souza de Azevedo *et al*. (2017), both these carbon sources were used to supplement the MRS medium. As a result, glucose is shown as the most important carbon source that *P. pentosaceus* ATCC 43200 metabolizes.

The antimicrobial activity of bacteriocin extract produced by *P. pentosaceus* grown in different culture media was assessed by agar well diffusion assay, performed on Mueller Hinton and MacConkey (*E. coli)* agar-media, after 24 h cultivation against *Enterococcus* spp. (strains Ent101, Ent104 and Ent711), *Listeria innocua* (strains Li2052 and Li2865) and *E. coli* ATCC 25922, taken as examples of Gram-positive and Gram-negative microorganisms. *L. innocua* strains were sensitive to the cultivation supernatant of GI-MRS and GS-MRS collected after 24 h (21-22 mm diameter), about 26% larger than supernatants of other media. *E. coli* was sensitive to the cultivation supernatant of G-MRS, S-MRS and I-MRS media (17.3 mm), on average 6.5% greater than Ent711 to the same supernatant (Table I). Figure 1 demonstrates the aspect of the inhibition halos of bacteriocin extract produced by *P. pentosaceus* in G-MRS after 24 h of cultivation when the assays were performed on Mueller Hinton and MacConkey agar-media.

The reproducibility of these results performed on Muller Hinton and MacConkey agar-media (Table I; Figure 1) was also assessed by comparing the activity

TABLE I - Antimicrobial activity (mm) of bacteriocin extract produced by *P. pentosaceus* ATCC 43200 cultivated in different media and collected at 24 h. The assays were performed on Mueller Hinton (*Enterococcus* and *Listeria*) and MacConkey (*E. coli*) agar-media

		Antimicrobial activity					
Time (h)	**Indicator strains**	**G-MRS**	**GS-MRS**	**GI-MRS**	**S-MRS**	**I-MRS**	**GSI-MRS**
24	*Enterococcus sp*. 101	12.10±0.14^{a}	12.05±0.07^{a}	12.00±0.02^{a}	11.60±0.14^{a}	11.00±0.02^{a}	13.45±0.07^{d}
	Enterococcus sp. 104	12.65±0.07^{b}	13.35±0.07^{b}	13.20±0.14^{c}	13.90±0.14^{b}	13.00±0.03^{b}	12.55±0.07^{c}
	Enterococcus sp. 711	15.40±0.14^{c}	12.05±0.07^{a}	12.00±0.02^{a}	16.40±0.14^{d}	16.60±0.14^{e}	12.00±0.02^{a}
	Listeria innocua 2052	16.50±0.01^{d}	22.00±0.04^{d}	20.05±0.07^{d}	16.00±0.11^{c}	15.00±0.04^{c}	16.00±0.04^{f}
	Listeria innocua 2865	18.00±0.02^{e}	20.00±0.03^{c}	21.35±0.07^{e}	17.00±0.04^{e}	16.00±0.05^{d}	13.90±0.14^{e}
	Escherichia coli ATCC 25922	16.60±0.14^{d}	12.10±0.14^{a}	13.00±0.05^{c}	17.00±0.04^{e}	18.45±0.07^{f}	12.50±0.01^{b}

Legend: G-MRS = glucose-based MRS medium, GS-MRS = glucose + sucrose-based MRS medium, GI-MRS = glucose + inulin-based MRS medium, S-MRS = sucrose-based MRS medium, I-MRS = inulin-based MRS medium, GSI-MRS = glucose + sucrose + inulin-based MRS medium. Mean values (n=3) ± standard deviations. Different letters in the same column mean that values significantly differ among them ($p < 0.05$).

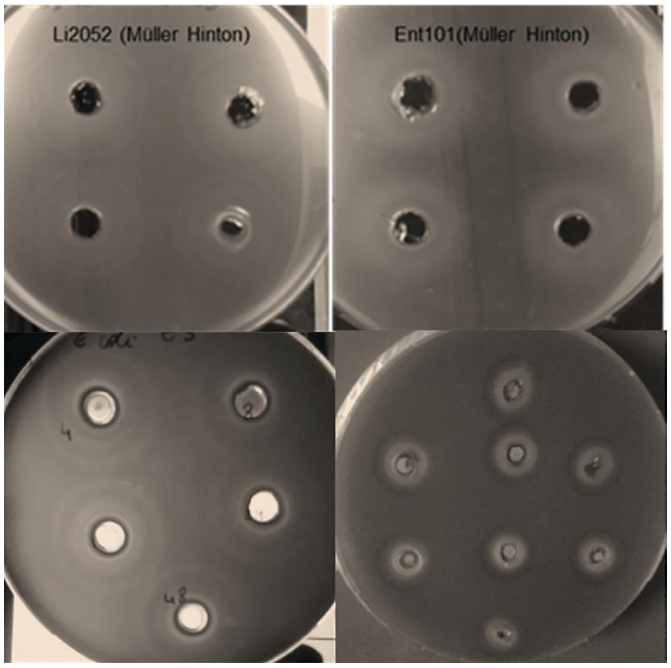

FIGURE 1 - Antimicrobial activity of bacteriocin extract produced by *Pediococcus pentosaceus* ATCC 43200 after 24 h of cultivation in G-MRS medium against *Listeria innocua* 2052 (Li2052), *Enterococcus* 101 (Ent101) and *Escherichia coli* ATCC 25922 (*E. coli*). Tests were performed by agar-well-diffusion assay carried out on Muller Hinton (Enterococcus and Listeria strains) and MacConkey (*E. coli*) agar-media. (A, C, E = indicator strain at OD 0.3); (B, D, F = indicator strain diluted 100 times from OD 0.3).

of these supernatants performed on BHI and TSB agar-media (Table II; Figure 2). It was evident the difference between the aspect and the diameters of the inhibition halos according to the agar-media used in the test. *Enterococcus* and *Listeria* strains were susceptible to the antimicrobial activity of supernatants (24 h) but the aspect and the diameters of the halos were not related to Muller Hinton and BHI agar-media. As expected, there was no antimicrobial activity against *E. coli* (Table II) when the test was performed on TSB agar-medium, since Gram-negative bacteria are naturally resistant to the action of bacteriocins produced by Gram-positive bacteria due to their outer membrane (Cao-Hoang, Marechal, Le-Thanh, 2008; Gyawali, Ibrahim, 2014). However, a questionable result could have been considered if this analysis had been carried out only on MacConkey agar-medium, since apparent inhibition halos against *E. coli* were seen only in MacConkey and were not seen in TSB agar-medium.

Among all strains evaluated, there was a significant difference in size of the inhibition halos according to the culture media used for the bacteriocin extract production (Table I; Table II), however, the most noteworthy observation that should be highlighted was the absence of inhibition halo against *E. coli* when the bacteriocin extracts were evaluated on TSB agar-medium.

A possible explanation for these results is the different agar-media composition. There are cations (Ca^{2+} and Mg^{2+}) and thymidine in the composition of Mueller Hinton agar-medium, components that are absent in BHI agar-medium, which may have somehow interacted with molecules occurring in the tested supernatants, affecting the obtained results (diameter of inhibition halo). MacConkey is a selective medium for Gram-negative bacteria and, unlike the TSB medium, there are bile salts, neutral red and crystal violet in its composition. In some

TABLE II - Antimicrobial activity (mm) of bacteriocin extract produced by *P. pentosaceus* ATCC 43200 cultivated in different media and collected at 24 h. The assays were performed on BHI (*Enterococcus* and *Listeria*) and TSB (*E. coli*) agar-media

		Antimicrobial activity					
Time (h)	**Indicator strains**	**G-MRS**	**GS-MRS**	**GI-MRS**	**S-MRS**	**I-MRS**	**GSI-MRS**
24	*Enterococcus sp*. 101	17.95±0.07^{b}	17.05±0.07^{d}	17.00±0.02^{e}	4.60±0.14^{a}	4.05±0.07^{a}	17.70±0.14^{c}
	Enterococcus sp. 104	16.15±0.21^{a}	16.10±0.14^{c}	16.00±0.01^{c}	7.15±0.21^{b}	6.00±0.01^{b}	16.15±0.21^{a}
	Enterococcus sp. 711	16.00±0.12^{a}	16.15±0.21^{c}	16.25±0.35^{d}	9.70±0.01^{d}	9.65±0.07^{f}	16.00±0.02^{a}
	Listeria innocua 2052	16.40±0.34^{a}	15.00±0.01^{b}	13.15±0.21^{a}	9.10±0.14^{c}	8.05±0.07^{c}	16.00±0.02^{a}
	Listeria innocua 2865	16.45±0.17^{a}	13.00±0.02^{a}	14.40±0.14^{b}	10.10±0.14^{e}	9.00±0.02^{e}	15.05±0.07^{b}
	Escherichia coli ATCC 25922	-	-	-	-	-	-

Legend: G-MRS = glucose-based MRS medium, GS-MRS = glucose + sucrose-based MRS medium, GI-MRS = glucose + inulin-based MRS medium, S-MRS = sucrose-based MRS medium, I-MRS = inulin-based MRS medium, GSI-MRS = glucose + sucrose + inulin-based MRS medium. Mean values (n=3) ± standard deviations. Different letters in the same column mean that values significantly differ among them ($p < 0.05$).

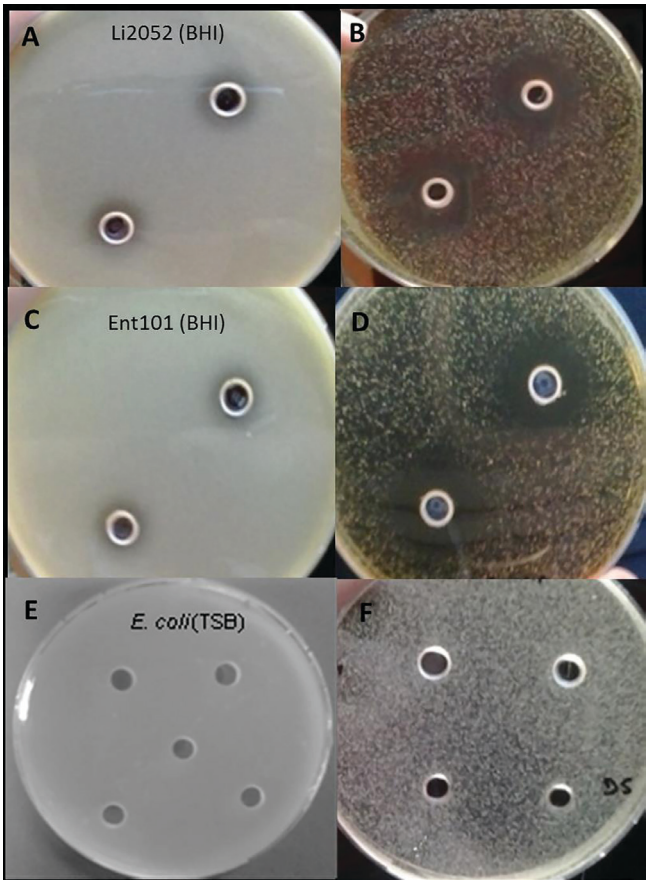

FIGURE 2 - Antimicrobial activity of bacteriocin extract produced by *Pediococcus pentosaceus* ATCC 43200 after 24 h of cultivation in G-MRS medium against *Listeria innocua* 2052 (Li2052), *Enterococcus* 101 (Ent101) and *Escherichia coli* ATCC 25922 (*E. coli*). Tests were performed by agar-well-diffusion assay carried out on BHI (Enterococcus and Listeria strains) and TSB (*E. coli*) agar-media. (A, C, E = indicator strain at OD 0.3); (B, D, F = indicator strain diluted 100 times from OD 0.3).

way, the tested supernatants could interact with one of these different components present in MacConkey agar-medium, hence producing false positive results as shown in Figure 2.

Currently, it is difficult to find comparison in the literature about using bioassays performed only in solid (agar) medium as presented in this work, meanwhile it is possible to find tests performed either in solid (agar diffusion assay) or in liquid (turbidimetry) media more easily. A major difficulty in bacteriocin research is obtaining accurate results using bioassays, which are based in inhibition activity produced in a sensitive microorganism (Rasch, Knochel, 1998; Papagianni *et al*., 2006).

Using the agar spot assay, a method also performed on agar-medium and very similar to agar well diffusion assay, Arena *et al*. (2016) identified strong inhibition effect of seventeen *Lactobacillus plantarum* strains according to the classification made by Gaudana, Dhanani and Bagchi (2010), showing inhibition halos of greater than 5 mm against the majority of food pathogens tested. The antimicrobial activity of the tested *Lb. plantarum* strains were mostly observed when they were grown on solid media, rather than brought into contact with pathogenic bacteria. To the performed well diffusion assay, the antimicrobial capability was confirmed when the cell-free supernatant (CFS) was concentrated ten-fold, thus indicating that a minimal concentration of antimicrobial compounds was required to sustain similar inhibition results (Arena *et al*., 2016).

The CFS may also include other molecules besides those secreted by bacteria such as medium components and/or intracellular compounds, which may be accidentally

released during CFS preparation (Arena *et al*., 2016). Despite this possibility, CFS are routinely used to preliminary screen for antimicrobial activity of bacteria by well diffusion method (Kassaa *et al*., 2014; Wang *et al*., 2014). This indicates that the inhibitory effects mainly depend on exudates, which are included in the CFS (Arena *et al*., 2016).

Other investigations observed that indicator cultures showed different sensitivity in agar and in liquid medium. The results of these investigations suggested a greater capability of CFSs to contrast pathogenic bacteria in liquid-medium in comparison to agar-medium (Saadatzadeh *et al*., 2013). Compared with *Lactobacillus curvatus*, *Listeria monocytogenes* and *Listeria seeligeri*, they were more sensitive to bacteriocin action on agar-medium than in liquid-medium and bacteriocins showed a greater activity against *Lb. curvatus* in liquid-medium than in agar-medium (Coventry *et al*., 1997). In another investigation with nine test-microorganisms used, only two of them, *Lb. curvatus* ATCC 51436 and *P. acidilactici* ATCC 25740, were sensitive to very low bacteriocin (nisin) concentrations and produced a linear type of response either in agar-medium (agar diffusion assay) or liquid-medium (turbidometric assay) (Papagianni *et al*., 2006).

The results presented in this work indicated different measurements of bacteriocin activity to the same test-microorganisms when the assays were performed in different solid medium (agar diffusion assay). These data suggest that any compared data in the literature must always be performed among identical methods, highlighting the need to choose carefully the agar-medium used in bacteriocin research.

ACKNOWLEDGMENTS

Financial support for this project was provided by FAPESP (grant 2016/06284-9 to RPSO) and CAPES (grant 1560096 to POSA).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Received for publication on 28th August 2017
Accepted for publication on 11th September 2017