Short communication: In vitro and in vivo probiotic potential of *Lactobacillus plantarum* B7 and *Lactobacillus rhamnosus* D1 isolated from Minas artisanal cheese

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ABSTRACT

Some *Lactobacillus* strains may contribute to the health of the host when administered in adequate concentrations, demonstrating their probiotic potential. In contrast, *Listeria monocytogenes* is a foodborne pathogen that can cause enteropathy, meningoencephalitis, abortion, and septicemia. The aim of this survey was to evaluate the in vitro and in vivo probiotic potential of *Lactobacillus plantarum* B7 and *Lactobacillus rhamnosus* D1, isolated from Minas artisanal cheese of the Serra da Canastra (Minas Gerais, Brazil), against *Lis. monocytogenes*. We submitted B7 and D1 to in vitro testing (antibiogram, tolerance to bile salts and artificial gastric fluid, and spot-on-lawn) and in vivo testing (relative weight gain in mice). Both *Lactobacillus* strains demonstrated in vitro inhibitory activity against *Lis. monocytogenes*, as well as sensitivity to antimicrobials and resistance to gastric acids and bile salts. In the in vivo assays, mice treated with D1 gained more weight than mice in the other groups. These results indicate that D1 could have higher probiotic potential than B7 because improvements in feed conversion may help animals fight infection.

Key words: *Lactobacillus*, probiotics, BALB/c, *Listeria monocytogenes*

Short Communication

Some *Lactobacillus* strains are considered probiotics; that is, they may confer health benefits on their host when administered in adequate amounts (FAO/WHO, 2002). Studies have already shown the capacity of *Lactobacillus* to improve the condition of digestive and

genitourinary systems, and to provide anticancer effects and metabolism regulation (Pandey et al., 2015). Among the health benefits of *Lactobacillus* administration are protection against infectious agents because of nutrient competition, competitive exclusion, direct antagonism, and immuno- and inflammatory modulation (Lebeer et al., 2008).

Listeriosis is a foodborne disease caused by consuming food contaminated with *Listeria monocytogenes*. This pathogen can be found in animal products, mainly dairy products such as raw milk and cheese (Sonner et al., 2018). There are 2 types of listeriosis. The noninvasive form is mild: clinical signs include gastroenteritis, fever, vomiting, abdominal pain, and diarrhea. The invasive form is more severe and can cause abortion, meningitis, meningoencephalitis, encephalitis, and septicemia; mortality rates range from 20 to 30% (Ryser, 2011).

In vitro and in vivo studies have shown that some *Lactobacillus* strains can inhibit the growth of *Lis. monocytogenes*. Ortiz-Rivera et al. (2017) observed anti-*Listeria* effects in meat and dairy products after *Lactobacillus* inoculation. Lukic et al. (2017) demonstrated the immunomodulatory activity of *Lactobacillus* in mice, resulting in protection against listeriosis. The inhibition of *Lis. monocytogenes* has also been observed in other studies evaluating the probiotic potential of lactic acid bacteria isolated from cheeses, including lactobacilli isolated from Minas artisanal cheeses (Costa et al., 2013; Sant'Anna et al., 2017). Acurcio et al. (2017) have also shown that increased weight gain in mice treated with *Lactobacillus* strains may decrease the severity of infections.

The aim of this study was to evaluate the in vitro and in vivo probiotic potential of 2 *Lactobacillus* strains against *Lis. monocytogenes*. The strains used were *Lb. plantarum* B7 and *Lb. rhamnosus* D1, isolated from Minas artisanal cheese produced in Serra da Canastra

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region, Brazil, and identified by Resende et al. (2011); and *Lis. monocytogenes* ATCC 15313.

The parameters established for evaluation of the in vitro probiotic potential of B7 and D1 were tolerance to bile salts and acidity (Silva et al., 2013), sensitivity to antimicrobials (Charteris et al., 1998), and inhibitory activity against *Lis. monocytogenes* by spot-on-lawn antagonism (Tagg et al., 1976). To establish a comparative pattern, we also tested *Lis. monocytogenes* against commercial probiotic samples: 6 *Lactobacillus casei* strains (AC, EL, IT, PA, VI, YA), *Lactobacillus paracasei* (CH), and *Lb. rhamnosus* GG (GG; Amara and Shibl, 2015).

For the in vivo assays, we obtained 70 4-week-old conventional male BALB/c mice weighing 15–17 g from the Biotério Central at the Universidade Federal de Minas Gerais (Belo Horizonte, MG, Brazil). The animals were kept in the Laboratório de Ecologia e Fisiologia de Microorganismos (Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil) in mini-isolators (ALE. MIL.01.03; Alesco, São Paulo, SP, Brazil) and placed on a ventilated shelf under temperature (22–24°C) and humidity (60–80%) control, in a day/night cycle of 12 h. Filtered water and solid food (Nuvilab, Curitiba, PR, Brazil) were offered ad libitum. All procedures in the study were performed following the requirements of Comissão de Ética no Uso de Animais at the Universidade Federal de Minas Gerais (CEUA) under protocol number 80/2015.

We performed a preliminary assay to determine the most suitable doses and route of inoculation to reproduce listeriosis in murine models. We divided the mice into 9 groups of 3, and each group received an inoculum (whole doses) of *Lis. monocytogenes*: 100 µL by oral gavage at 10^9 , 10^8 , and 10^7 cfu; 100 µL intraperitoneally at 10^7 , 10^6 , and 10^5 cfu; or 50 µL intravenously at 10^7 , 10^6 , and 10^5 cfu. These values were based on the findings of previous studies (Campion et al., 2013; D'Orazio, 2014; Quereda et al., 2016). All animals were evaluated for 15 d for mortality, morbidity, and general clinical signs of listeriosis.

Later, we divided a new set of mice into 3 groups of 14; each received the following treatments (whole doses) by gavage each day for 2 wk: 100 µL of inoculum of B7 at 10^8 cfu, 100 µL of inoculum of D1 at 10^8 cfu, or 100 µL of 0.9% saline (control group). On d 11 of treatment, all animals were infected with *Lis. monocytogenes*, according to the dose and route of inoculation determined in the previous assay (10^6 cfu intravenously). Throughout the 2 wk, the mice were weighed every 48 h.

GraphPad Prism 7.0 software (GraphPad software, San Diego, CA) was used for all statistical analyses. We compared the means of the inhibition halos from spot-on-lawn antagonism tests using the Kruskal–Wallis

test, followed by a Dunn post-test at a 5% significance level. In the in vivo studies, we analyzed the relative weight gain results by 2-way ANOVA and compared the findings using a Sidak test at 5% significance.

Table 1 shows the tolerance of B7 and D1 to bile salts and artificial gastric fluid. Both strains may be classified as tolerant to acid and moderately tolerant to oxgall (Acurcio et al., 2014). Studies of the growth inhibition of lactic acid bacteria isolated from Minas artisanal cheese have shown percentages varying from –24 to 19.68% with artificial gastric fluid and from –1 to 89.73% with oxgall (Costa et al., 2013; Sant'Anna et al., 2017). Although B7 and D1 showed moderate tolerance to bile, Ruiz-Moyano et al. (2008) have suggested that resistance could increase when the microorganisms are previously exposed to acid, as occurs in the oral administration of probiotics. According to Lin et al. (2006), some microorganisms tend to express resistance mechanisms when they are previously exposed to sub-lethal injuries.

The sensitivity pattern of B7 and D1 to antimicrobials is also described in Table 1. The results for both strains resembled those of other studies of the antimicrobial susceptibilities of *Lactobacillus* strains (Costa et al., 2013; Sant'Anna et al., 2017). Phenotypically, both strains had an appropriate antimicrobial sensitivity pattern, but it would be important to search for resistance genes to more reliably characterize the resistance of B7 and D1 to antimicrobials.

The mean diameters of inhibition halos against *Lis. monocytogenes* produced by B7, D1, and other probiotic strains *Lis.* are shown in Figure 1. All lactobacilli were able to inhibit *Lis. monocytogenes*, and we observed

Table 1. Survival after 12 h and antimicrobial susceptibility profile of *Lactobacillus plantarum* B7 and *Lactobacillus rhamnosus* D1 isolated from Minas artisanal cheese of the Serra da Canastra

Analysis	<i>Lactobacillus</i> strain	
	B7	D1
Survival after 12 h (%)		
Bile salts (0.3% oxgall)	57.95	46.10
Artificial gastric fluid (pH 2.0, pepsin 3 g/L) ¹	16.65	1.06
Antimicrobial sensitivity ¹		
Ampicillin (10 µg)	S	S
Cefoxitin (30 µg)	R	R
Ceftriaxone (30 µg)	MS	S
Ciprofloxacin (5 µg)	S	S
Chloramphenicol (30 µg)	S	S
Streptomycin (10 µg)	R	R
Gentamicin (10 µg)	R	R
Penicillin (10 µg)	S	S
Tetracycline (30 µg)	S	S
Vancomycin (30 µg)	R	R

¹S = sensitive; MS = moderately sensitive; R = resistant.

no statistical difference between the means inhibition halos of the tested strains (B7 and D1) and the other probiotics. The only significant differences we observed were between probiotic strains: CH and VI ($P < 0.05$), EL and VI ($P < 0.05$), and GG and EL ($P < 0.05$). Sip et al. (2012) concluded that 284 of 800 (35%) lactic acid bacteria samples isolated from Golka cheese, produced in Poland, inhibited the growth of *Lis. monocytogenes*. Likewise, 31.9% of the 699 lactic acid bacteria samples isolated from Sicilian cheeses could interfere with the growth of this pathogen (Macaluso et al., 2016).

In the in vivo experiment, we did not observe clinical signs of listeriosis in mice infected orally or intraperitoneally during the initial 15 d of observation, regardless of the inoculum concentration. Likewise, mice challenged with 10^5 cfu of *Lis. monocytogenes* using an intravenous route did not show any perceptible clinical changes. The resistance of mice to listeriosis can be explained by the weak affinity between host epithelial receptors (E-cadherin and MET) and *Lis. monocytogenes* surface proteins called internalins, especially InlA and InlB (D'Orazio, 2014). In addition, 90% of the *Lis. monocytogenes* cells that reach the bloodstream are removed from circulation and phagocytosed by Kupffer cells in the liver (Llorente and Schnabl, 2016). Becattini et al. (2017) also highlighted the importance of bacteria of the order Clostridiales (present in the intestinal microbiota of mice) in inhibiting the growth of *Lis. monocytogenes*. The pathogenicity of the *Lis. monocytogenes* strain may also have hindered the establishment of disease. Liu et al. (2003) demonstrated that after challenging mice intraperitoneally with different strains of this pathogen, *Lis. monocytogenes* ATCC 15313 had the highest median lethal dose ($>1.2 \times 10^{11}$ cfu).

Mice in the groups that received intravenous inoculum with 10^6 and 10^7 cfu of *Lis. monocytogenes* developed clinical signs that resulted in death in some cases. In the first group, all mice showed clinical signs of listeriosis on d 2 after inoculation. Two mice died the next day, and the surviving mouse showed resolution of clinical signs on d 6. In the second group, 2 mice presented clinical signs of listeriosis 24 h after infection, leading to death on d 2. The other mouse began to present clinical signs on d 2, resulting in death on d 3. We observed nonspecific clinical signs such as apathy, anorexia, matted fur, conjunctivitis, and tremors. These findings were consistent with those of Hardy et al. (2015), 4 d after an intravenous challenge of mice with *Lis. monocytogenes* (10^5 cfu). Based on the results, we chose an intravenous dose of 10^6 cfu as our experimental model of infection. Among the treatments tested, it showed the ability to reproduce less intense disease in BALB/c mice than the others.

Figure 1. Inhibition halos (mm, mean $\pm$ SD) formed by *Lactobacillus casei* (strains AC, EL, IT, PA, VI, and YA), *Lactobacillus paracasei* (CH), *Lactobacillus rhamnosus* (D1 and GG), and *Lactobacillus plantarum* (B7) against *Listeria monocytogenes* ATCC 15313 in spot-on-the-lawn test. * $P < 0.05$, Kruskal–Wallis test, followed by Dunn's post-test.

The results for the relative weight gain of mice treated with B7, D1, and controls, before and after challenge with *Lis. monocytogenes*, are shown in Figure 2. The weight gain observed in animals that received D1 was not connected to the anti-*Listeria* effect, and was related to a potential probiotic effect that could be exhibited by other lactic acid bacteria. During the pretreatment period, we found no statistical differences between the development of the mice in the control group and the mice in the B7 group. However, after the challenge we found greater weight loss in the mice in the B7 group. Frizzo et al. (2010) observed similar results when treating mice with *Lb. plantarum* and challenging them with enteropathogens. According to Million et al. (2012), some strains of *Lb. gasseri* and *Lb. plantarum* present an antiobesity effect in the host organism, which results in weight loss. In contrast, the D1 group in our study showed greater weight gain compared with the control group during bacterial administration. The significant difference between the groups remained even after weight loss resulting from inoculation with *Lis. monocytogenes*. This finding suggests that the administration of D1 can improve physical condition, which may result in greater tolerance of some diseases.

The *Lb. plantarum* B7 and *Lb. rhamnosus* D1 strains isolated from Minas artisanal cheese produced in the Serra da Canastra region showed resistance to acid and bile salts, antimicrobial sensitivity, and antagonistic effects against *Lis. monocytogenes*. Both strains had

Figure 2. Relative weight gain (%) of BALB/c mice ($n = 14$), control (CTL) or treated with *Lactobacillus plantarum* B7 and *Lactobacillus rhamnosus* D1 (10^8 cfu inoculum orally for 4 wk) before and after challenge with *Listeria monocytogenes* ATCC 15313 (10^6 cfu inoculum intravenously at d 11). * $P < 0.05$, 2-way ANOVA, followed by Sidak's post-test. Error bars indicate SD.

satisfactory in vitro probiotic potential and could be tested in future in vivo assays. The mice in the D1-treated group showed greater weight gain during pre-treatment and after the *Lis. monocytogenes* challenge. Therefore, *Lb. rhamnosus* D1 may be the most interesting microorganism for use as a probiotic because of its performance in in vivo and in vitro tests.

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