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Crude extracts of metabolites from co-cultures of lactic acid bacteria are highly antagonists of *Listeria monocytogenes*

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ABSTRACT

Listeria monocytogenes is a pathogen difficult to control, due to its resistance to extreme conditions. The antimicrobial activity of a mixture of metabolites produced by lactic acid bacteria was evaluated against *L. monocytogenes*. Bacterial combined cultures in 1:1 ratio of *Lactobacillus plantarum* and *Weissella cibaria* (treatment LP + WC) and mixtures in ratio 1:1:1 of *Lactobacillus brevis, L. plantarum,* and *W. cibaria*, (treatment (LB + LP + WC) were grown by discontinuous fermentation, at 32 °C for 48 h. At 1, 2, 6, 12, 24 and 48 h of fermentation, samples were taken, the biomass was separated from the metabolites, and the antimicrobial activity of the metabolites was measured *in vitro* against *L. monocytogenes*. For comparison, experimental data published in the literature corresponding to monocultures of *L. brevis* (L.B), *L. plantarum* (LP) and *W. cibaria* (WC) were used. The antimicrobial activity was measured by a surface diffusion technique using absorbent paper discs impregnated with 60 μl from each metabolite and placed on the TSA agar surface (36 °C, 24 h). The metabolites from the microbial mixtures showed statistical differences with respect to their respective monocultures. With the treatment (LP + WC) an inhibition diameter of 2.54 cm was obtained at 12 h of fermentation, this value was higher than those obtained in the monoculture LP (2.19 cm), and WC (2.44 cm), during the same period. In the mixture (LB + LP + WC) during the first 12 h of fermentation, the antimicrobial activity was higher (2.12–2.28 cm) than the antimicrobial activity of the monoculture LB (1.66–2.23 cm). The use of metabolites from the co-culture of *L brevis, L. plantarum* and *W. cibaria* under the evaluated conditions, potentiate the antimicrobial activity of *L. brevis* against *L. monocytogenes,* therefore, they are promising in bio-preservation.

1. Introduction

At least 90% of reported cases of *Listeria monocytogenes* infection have been linked to the consumption of contaminated food (Tao et al., 2017). This pathogen frequently affects seniors, pregnant women, new-borns and immunodeficient individuals (Campillo et al., 2017) and can cause fatal cases in of up to 30% of patients in these population groups (Rawool et al., 2016). The presence of this microorganism is challenging to control; in comparison with other foodborne pathogens, *L. monocytogenes* is resistant to extreme conditions such as high salt concentrations (Rawool et al., 2016), low pH (Changcheng et al., 2017) and low temperatures; therefore there is a high risk of contamination in refrigerated and frozen foods (Tao et al., 2017). Several strategies have been implemented to control this microorganism, including an emerging non-thermal technology, which involves the use of electric field pulses. However, its effectiveness remains uncertain and requires more detailed studies (Changcheng et al., 2017). On the other hand, thermal treatments have been shown to be effective in inactivating both pathogenic microorganisms such as *L. monocytogenes*, as deteriorating microorganisms in food; however, they often affect the organoleptic characteristics of food products.

Besides the effect in flavor and aroma produced by lactic acid bacteria (LAB) in fermented foods, the acid pH produced by their presence, which also helps in food preservation (Axelsson et al., 1989; Hernández-Mendoza et al., 2007). These bacteria have antimicrobial activity due to the final products of their metabolism such as lactic acid, acetic acid, hydrogen peroxide, diacetaldehyde, reuterin, and bacteriocins (Hugas, 1998). Therefore, lactic acid bacteria have been considered as an

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alternative to the use of chemical preservatives, which have allowed a decreasing in the intensity of thermal treatments and have shown antimicrobial activity against *L. monocytogenes* (García-Salazar et al., 2016; García-Gonzales et al., 2017).

Anacarso et al. (2014) evaluated in salmon fillets, the antimicrobial activity of bacteriocins produced by *L. pentosus* 39 in monoculture against *L. monocytogenes*, finding a decreasing in the pathogen quantity. While other studies have reported antimicrobial activity of *Lactobacillus plantarum* DSM1055, CH3, SAU96 and *Lactobacillus brevis* in monoculture against strains of *L monocytogenes* (Hartmann et al., 2011; Lacerda et al., 2013). However, an increase in the antimicrobial activity of mixtures of microbial cultures (co-culture) of lactic acid bacteria has been documented (Calasso et al., 2013), and it has even been found that lactic acid bacteria that do not produce bacteriocins in monoculture can produce them in co-culture (Maldonado et al., 2004). The aim of this study was to evaluate the kinetics of antimicrobial activity against *L. monocytogenes* from mixtures of metabolites produced by the simultaneous fermentation of three lactic acid bacteria, *Weissella cibaria*, *L. brevis,* and *L. plantarum* and from the mixture of *W. cibaria* and *L. plantarum.*

2. Materials and methods

2.1. Cultivation of microorganisms and measurement of inhibitory activity

Three strains of lactic acid bacteria were used as follows: *L. brevis, L. plantarum,* and *W. cibaria.* The first two strains were isolated from yellow pitahaya (*Selenicereus megalanthus Haw)* fruits and the last one, from the bovine ruminal liquid in previous works of Valencia-Hernández et al. (2016); and Serna et al. (2010), respectively. The pathogenic microorganism used to measure antimicrobial activity was *L. monocytogenes* ATCC 13932®.

Bacterial strains were grown in co-culture by discontinuous fermentation by triplicate. Fermentations of the bacteria were carried out in a 1:1 ratio of *L. plantarum* and *W. cibaria* treatment (LP + WC) and in a 1:1:1 ratio of *L. brevis, L. plantarum,* and *W. cibaria*, treatment (WC + LB + LP). Erlenmeyer flasks of 500 ml with a working volume of 300 ml were used. The fermentations were carried out for 48 h at 32 °C. The methodologies proposed by Serna et al. (2010) and García-Salazar et al. (2016) were followed for the fermentation. After 1, 2, 6, 12, 24 and 48h of fermentation, 5 mL of the fermented medium were taken. In order to separate the biomass from the metabolites, the sample was centrifuged for 15 min at 5000 x g (Eppendorf Centrifuge-5804R, Germany®), the supernatant was filtered twice, initially with a 0.45 μm diameter filter, and subsequently, with a 0.2 μm diameter filter. The filtrate was used to perform microbial activity tests.

Antimicrobial activities of biomass and metabolites were compared with previous studies reported by García-Salazar et al. (2016) and García-Gonzales et al. (2017).

The evaluation of the antimicrobial activity was carried out using the surface diffusion technique proposed by Ryan et al. (1996) with some modifications. Three plates of Trypticase Soy Agar (TSA) with a thickness of 5 mm, substrate were used for each treatment that allowed the growth of the pathogenic microorganism *L. monocytogenes*. Plates were seeded with the pathogen using surface seeding. Separately, absorbent paper discs were impregnated with 60 μl from each metabolite and placed on the TSA agar surface. The plates were incubated at 36 °C for 24 h, and photographs of growth inhibition were taken. The images were processed in an image analysis software (Image j 1.40 g, Wayne Rasband, National Institutes of Health, USA), and the Feret diameters were determined.

2.2. Experimental design

An ANOVA was carried out and Tukey's multiple range test was used to compare the mean values of treatments using RStudio Version 0.99b®, 2016 statistical software, where the experimental data of the treatments (LP + WC) and (LB + LP + WC) were analyzed and compared. The response variable was the Feret diameter of *L. monocytogenes* growth inhibition.

3. Results

3.1. Antimicrobial activity

In Table 1 the Feret diameter values of the treatments (LP + WC) and (LB + LP + WC), and, the values extracted from the literature corresponding to the antimicrobial activity against *L. monocytogenes* of the respective monocultures are presented. In addition, this table shows the statistically significant differences presented among treatments.

In the mixture (LB + LP + WC) until the 12th hour, the values of antimicrobial activity were higher than those of LB monoculture. Nevertheless, Figs. 1 and 2 proved that the highest antimicrobial activity against *L. monocytogenes* is obtained with the WC monoculture. The combination of three strains (LB + LP + WC) of bacteria, did not potentiate the antimicrobial activity either of LP nor of WC.

3.2. Effect of fermentation time on antimicrobial activity

Statistically significant differences were found among fermentation times. The treatments presented a decrease in the antimicrobial activity during the fermentation (Figs 1 and 2). However, the mixture of metabolites (LP + WC) showed less variation in the antimicrobial activity against *L. monocytogenes* compared with their respective monocultures (Fig. 1).

The treatment (LP + WC) showed the statistical difference with WC (Table 1) in all the evaluated fermentation times and (LP + WC), with a statistical difference with the LP treatment only in the 12th hour of fermentation. The treatment (LB + LP + WC) always presented a difference with WC. In the 24–48 h, there were no statistical differences among treatments.

It was observed that in Table 1, the most significant inhibition diameters were presented at 12h of fermentation with the mixed culture. With the LP and WC monocultures, the largest inhibition diameters were presented at 6 h of fermentation. The mixture of lactic acid bacteria LB + LP + WC favored the antimicrobial effect against the pathogen *L. monocytogenes* compared to the antimicrobial activity of LB, which is the monoculture that reports the literature with less activity against this pathogen.

Table 1
Feret diameters (cm) obtained from mixtures of lactic acid bacteria metabolites against *L. monocytogenes.* Different letters in columns represent significant differences among treatments. LP and WC values correspond to experimental data from García-Gonzales et al. (2017), and the LB data corresponds to García-Salazar et al. (2016).

Time (h)	LB^{c}	LP^{a}	WC^{bc}	LP + WC^{bc}	LB + LP + WC^{ab}
1B	2.23 ± 0.43	2.36 ± 0.22	1.60 ± 0.04	2.27 ± 0.19	2.23 ± 0.26
2A	2.22 ± 0.25	2.49 ± 0.18	2.31 ± 0.37	2.18 ± 0.24	2.25 ± 0.08
6AB	1.66 ± 0.29	2.81 ± 0.43	2.73 ± 0.31	2.31 ± 0.13	2.12 ± 0.23
12AB	2.10 ± 0.32	2.19 ± 0.13	2.44 ± 0.23	2.54 ± 0.27	2.28 ± 0.17
24C	1.84 ± 0.20	1.94 ± 0.13	1.57 ± 0.08	1.66 ± 0.09	1.90 ± 0.04
48C	1.75 ± 0.10	1.69 ± 0.07	1.56 ± 0.14	1.72 ± 0.11	1.72 ± 0.04

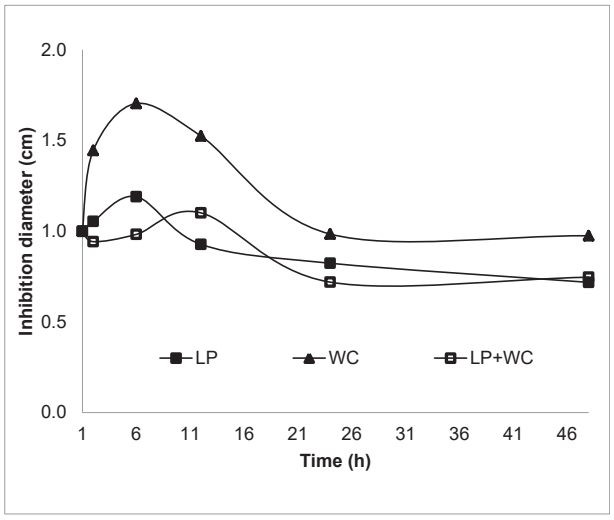

Fig. 1. Standardized graph of antimicrobial activity against *L. monocytogenes* with *W. cibaria* and *L. plantarum* (treatment LP + WC). WC and LP monoculture controls correspond to literature data (García-Salazar et al., 2016; García--Gonzales et al., 2017).

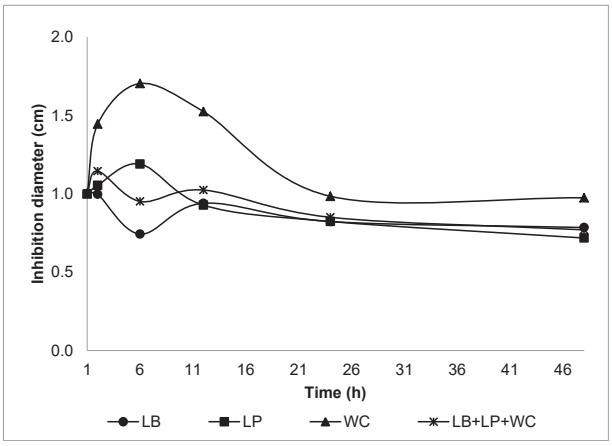

Fig. 2. Standardized graph of antimicrobial activity of the mixture of metabolites from *W. cibaria*, *L. brevis* and *L. plantarum* (treatment (LB + LP + WC). The WC, LB and LP monoculture controls correspond to literature data (García-Salazar et al., 2016; García-Gonzales et al., 2017).

4. Discussion

Three lactic acid bacteria were used in this study, *L. brevis* and *L. plantarum* isolated from a plant source and *W. cibaria* isolated from an animal source. Early, our research group reported the antimicrobial and fungistatic capacities of these bacteria against pathogens of fruit and animals destined for human consumption (Valencia-Hernández et al., 2016). The antimicrobial and fungistatic activities of these bacteria were attributed to the production of bacteriocins of very low molecular weight and the production of organic acids. The results permitted the development of bioproducts designed for the prevention of diseases in animals and plants of human consumption (patent resolution N ° 82497 and located NC 2016-0000370 of the Super Intendancy of Industry and Commerce, Colombia, respectively). In the present paper, the behavior of the metabolites from a mixed culture produced under co-cultivation by these microorganisms against a highly relevant pathogen in the food industry such as *L. monocytogenes* is described.

Several studies have been reported where the antimicrobial activity of different monocultures against *L. monocytogenes* being evaluated. Asurmendi, García, Pascual, and Barberis (2015) evaluated to the anti-listeria effect of monocultures of *L. plantarum*. The antimicrobial activity of *L. plantarum* against *L. monocytogenes* oscillated between 1.80 ± 1.8 to 2.00 ± 1.6 cm. In a study, the evaluation of the inhibitory effect of two strains of *L. plantarum* against two strains of *L. monocytogenes* was carried out by Hartmann et al. (2011), where the strain *L. plantarum* DSM1055 (obtained from a collection of microorganisms) did not show inhibition against any of the two strains of *L. monocytogenes*, and the strain *L. plantarum* IDE0105 (isolated from dried sausage), presented the largest inhibition diameter against the two strains of *Listeria* (0.6 cm). In the study carried out by Lacerda et al. (2013), the inhibition of some BAL against *L. monocytogenes* was evaluated, among them, two strains of *L. brevis* (FFC199 and SAU105, obtained by isolation of fermented foods) and three strains of *L. plantarum* (CH3 and CH41 isolated from cocoa (*Theobroma cacao* L.) fermentation and SAU96, isolated from sausage). It was found that *L. brevis* SAU105 and *L. plantarum* CH41 did not show any inhibitory effect, while the other strains presented an inhibition diameter which ranged from 0.1 to 0.2 cm. Also, Sakaridis et al. (2012) isolated some LAB from the poultry channel, and evaluate them against two pathogens that affect this type of meat, including *L. monocytogenes*; results showed that there was a growth inhibition of the pathogenic microorganism. When comparing results reported by others with the results contained in Table 1, where mixtures of *L. plantarum* and *L. brevis* were used. The observation on antimicrobial activity of the present study was significantly higher.

It should be considered that an increase in the antimicrobial activity of the mixture (LB + LP + WC) in relation to the LB monoculture that produces less antimicrobial activity against *L. monocytogenes,* which can be explained by the fact that the presence of more than one microorganism in the substrate generates a stress condition, which promotes its antimicrobial activity. Microbial stress causes bacteria to overexpression of the bacteriocin-like proteins, which have antimicrobial activity (Calasso et al., 2013). In addition, an increasing in the antimicrobial activity of LP + WC at 12h and LB + LP + WC from 1h to 12h, can be explained by the presence of *L. plantarum*, since it has been demonstrated that the production of plantaricin, which is a bacteriocin produced by *L. plantarum* is regulated by genes whose expression depends on external stimuli such as co-culture with other strains, which activates the *quorum sensing* mechanisms (Maldonado et al., 2004).

According to Guerrero et al. (2011), when there are more than two strains of microorganisms in the same space, a biocontrol condition of one strain can be presented over another(s) in which multiple modes of action are included; one of which is competition for nutrients used for development of essential functions in microorganisms such as respiration, nutrition, and others is competition for space. This can explain the behavior of the mixture (LB + LP + WC) versus the LP and WC monocultures. Subsequently, Layton et al. (2011) affirmed that this competition can hinder the inhibitory effect of the pathogens.

With the mixtures of metabolites, the best measures of the inhibition diameters were presented at 12h of fermentation and the LP and WC monocultures at 6h of fermentation, this can be explained by the competition exerted by the strains for having access to space and the nutrients of the substrate, this competence turns out to be relevant for vital processes (Guerrero et al., 2011; Layton et al., 2011).

The lactic acid bacteria in monoculture and mixture can generate secondary metabolites such as lactic acid, acetic acid, and ethanol during fermentation (Abdel-Rahman et al., 2013), which will depend on the homofermentative or heterofermentative nature of these bacteria (Preciado et al., 2013). Proteins or peptides with bactericidal action called bacteriocins can also be produced within the secondary metabolites, which is attributed to the antimicrobial capacity against *L. monocytogenes* (Beshkova and Frengova, 2012). There are different models that explain the mode of action of bacteriocins, however, and despite having some structural differences among them, the most recognized mode of action is

the formation of pores and ion channels in the cytoplasmic membrane of target cells, which modifies the plasma membrane permeability (Moll et al., 1999). In future research, it is important to evaluate the exact mechanism by which the mixture of metabolites of *L. brevis*, *L. plantarum,* and *W. cibaria* potentiate the inhibitory activity of *L. brevis* against pathogenic microorganisms such as *L. monocytogenes*.

The mixture of lactic acid bacteria LB + LP + WC favored the antimicrobial effect against the pathogen *L. monocytogenes* compared to the antimicrobial activity of LB, which was the monoculture that reported less activity against this pathogen. Therefore, it could be affirmed that the use of LB + LP + WC is a viable option as a bio-preservative in the food industry.

In all treatments from 24 h of fermentation, the antimicrobial activity against *L. monocytogenes* or decreased or stabilized, this is explained because bacteriocins are produced at baseline levels and become a signal and/or response in stress conditions, such as the presence of a pathogen (Riley and Wertz, 2002). Also, because bacteriocins may play a defensive role and act to inhibit the growth of *L. monocytogenes*, or the bacteriocins may serve as anti-competitors enabling the invasion of a strain into an established microbial community.

5. Conclusion

From this study it is concluded that the metabolites from the Co-culture of *L. brevis, L. plantarum,* and *W. cibaria* potentiate the action of *L. brevis*, which is a lactic acid bacterium with less antimicrobial activity against *L. monocytogenes*; therefore, the co-culture of certain species of lactic acid bacteria could be used in food bio-preservation with the aim to combat a pathogen so resistant to extreme conditions as *L. monocytogenes*.

Declarations

Author contribution statement

Liliana Serna-Cock, Cristobal N. Aguilar: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

María Rojas-Dorado, Diana Ordoñez-Artunduaga, Angela García-Salazar, Estefanía García-González: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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