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Effects of fermentation substrates and conservation methods on the viability and antimicrobial activity of *Weissella confusa* and its metabolites

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Abstract Lactic acid bacteria produce metabolites with antagonistic activity against other bacteria. However, growth conditions and conservation methods may reduce the viability and antimicrobial activity of lactic acid bacteria. This study evaluated the effects of fermentation substrate, lyophilization (freeze-drying) and refrigeration on the viability and antimicrobial activity of *Weissella confusa* strain and its metabolites against pathogens responsible for bovine mastitis. *W. confusa* strain was grown in MRS broth and milk supplemented with yeast extract and glucose (MYEG). The collected fractions were preserved by lyophilization or under refrigeration at 4ºC. Every seven days, the viability of *W. confusa* strain and the stability of its metabolites were evaluated against *Staphylococcus aureus* and *Streptococcus agalactiae* by disc diffusion assays. In both fermentation substrates, the combination of lyophilized strain and metabolites retained antimicrobial activity against the two pathogens for 42 days. Also, *W. confusa* strain retained adequate viability and antimicrobial activity when grown in MYEG and stored under refrigeration conditions. It was concluded that MYEG and refrigeration are acceptable low cost options to preserve the viability of *W. confusa* for its potential commercial use in the prevention and treatment of bovine mastitis.

Keywords: conservation, inhibition, lactic acid bacteria, lyophilization

INTRODUCTION

Weissella spp. are gram-positive bacteria (Björkroth et al. 2002) found in diverse habitats. Bacteria of this genus are able to produce antimicrobial compounds including lactic acid, hydrogen peroxide, diacetyl, and bacteriocins (Srionnual et al. 2007; Espeche et al. 2009). Srionnual et al. (2007) purified a bacteriocin produced by *Weissella cibaria* 110, active against *Lactobacillus sakei* and *Weissella paramesenteroides.* Espeche et al. (2009) isolated *W. paramesenteroides* in milk from healthy cattle that showed antagonistic effect against *Streptococcus dysgalactiae* ATCC 27957 and *Escherichia coli.* More recently, Serna-Cock et al. (2010) isolated *W. confusa* strain with antibacterial properties against *Staphylococcus aureus* and *Streptococcus agalactiae*.

Previous reports demonstrated the potential application of lactic acid bacteria and the *Weissella* genus for the prevention and control of bacterial pathogens (Tatsadjieu et al. 2009) including *S. aureus* and *S. agalactiae* (Ryan et al. 1998; Twomey et al. 2000; Lee, 2005; Serna-Cock et al. 2010), the most important pathogens responsible of bovine mastitis (Olde Riekerink et al. 2008, Capurro et al. 2010). It has been suggested that the continuous application of active *Weissella* spp strain and its metabolites to the udder of dairy cows, is a natural alternative to antibiotics for the prevention and control of

mastitis, since antibiotics can generate long term resistance and negatively affect milk production and quality (Barkema et al. 2006, Rodríguez-Noriega et al. 2010).

However, the evaluation of different conservation methods able to maintain viable strain and preserve antimicrobial activity is still needed (Otero et al. 2007). Low temperature storage (*e.g.*, freezing/cooling) and lyophilization (freeze-drying) are common methods used for the long term storage of viable strain (Schoug et al. 2006). Lyophilization is the most widely used method for the preservation of lactic acid bacteria over long periods of time (Miyamoto-Shinohara et al. 2006, Otero et al. 2007). However, freezing causes osmotic stress (Koch et al. 2008), dehydration (Rault et al. 2010), and cell membrane damage (Jagannath et al. 2010), resulting in loss of metabolic activity and cell viability (Conrad et al. 2000). Streit et al. (2007) reported a positive effect on the viability of frozen *Lactobacillus bulgaricus* CFL1 when pre-acidifying the fermented broth. The survival of lyophilized *Lactobacillus acidophilus* improved when exposed to lower water activity and oxygen level below 4% before freezing (Kurtmann et al. 2009).

The purpose of this work was to evaluate the effect of refrigeration and lyophilization, and type of fermentation substrate on the viability and antimicrobial stability of *W. confusa* strain and its metabolites against *S. aureus* and *S. agalactiae*. The long term goal driving this research is to generate cost-effective natural methods for the control and prevention of bovine mastitis by direct application of *W. confusa* strain and antimicrobial compounds to the udder of dairy cows.

MATERIALS AND METHODS

A cryo-preserved *W. confusa* strain effective against *S. aureus* and *S. agalactiae* was used. The strain was isolated from the ruminal liquid of Hartón del Valle cows (native Colombian breed) and selected from a group of 11 lactic acid bacteria based on its large lactic acid production (more than 12 gL^{-1} lactic acid), as reported by Serna-Cock et al. (2011). Bacterial growth was done in two separate fermentation substrates: (1) milk containing yeast extract and glucose (MYEG), and (2) commercial MRS broth (De Man et al. 1960). The MYEG substrate was prepared by mixing skim milk powder (11% w/v), yeast extract (1% w/v), and D(+)-glucose monohydrate (6% w/v) in distilled water and then sterilized at 121ºC for 15 min (Serna-Cock et al. 2010). Skim milk powder was used as a low cost alternative providing nitrogen and vitamins for the growth of *W. confusa* strain (Serna-Cock et al. 2010).

Fermentations were done in four 1000 mL Erlenmeyer flasks (2 flask/substrate, 600 mL effective volume) without aeration, under continuous agitation using an orbital shaker (model 5000I, VWR, USA) set at 33ºC and 100 rpm for 4 hrs. In each case, the initial inoculum was 10% of the substrate volume. Fermentation was adjusted to pH 6.0 using NaOH 4M. Specific methods were selected based on preliminary studies by Serna-Cock et al. (2010), who evaluated the kinetics of *W. confusa* grow and its antimicrobial activity against strains of *S. aureus* and *S. agalactiae* in commercial and milk substrates. After incubation, two fractions were obtained for each substrate: fraction Biomass + Metabolites (B+M) constituted by the fermentation substrate containing *W. confusa* strain and metabolites, and fraction Biomass (B) containing *W. confusa* strain. Fraction B+M was stored in 2 ml sterile vials (1 ml per vial, 40 vials), where half of the samples were refrigerated at 4ºC and half lyophilized at -20ºC (0.120 mB absolute pressure, -50ºC condenser temperature).

For fraction B, the fermented substrate from each broth (MRS or MYEG) was poured into 45 mL centrifuge tubes and centrifuged for 30 min at 2860 x G (Model 5804R, Eppendorf, CITI, Germany). Then, the supernatant was removed and the precipitate in each tube was re-suspended in 1 mL physiological water (0.9% NaCl), gently agitated, and centrifuged for 5 min at 2860 x G and the supernatant discarded. Half of the tubes from each substrate were re-suspended in 1mL sterile distilled water, poured in 2 ml sterile vials, and lyophilized at -20ºC. The other half was suspended in 1 mL broth (MRS or MYEG), poured in 2 mL sterile vials (20 vials per broth) and refrigerated at 4ºC. The following nomenclature was used: LB: lyophilized biomass, LB+M: lyophilized biomass and metabolites, RB: refrigerated biomass, RB+M: refrigerated biomass and metabolites.

Viable *W. confusa* strain in the biomass samples was determined for each substrate (MYEG *vs.* MRS) and conservation method (lyophilization *vs.* refrigeration) in a weekly basis for six weeks. Once a week, a sample (1 mL) per treatment was collected, serially diluted in peptone water and pour plated in MRS

agar Petri plates. Duplicate plates per dilution were incubated at 32ºC and colony forming units (cfu) counted after 24 hrs (ICMSF, 2000).

For six weeks, a weekly test for antimicrobial activity using the disc diffusion assay (Ryan et al. 1996) was done against *S. aureus* (ATCC 25923^{TM}) and *S. agalactiae* (ATCC 13813^{TM}) using Petri dishes containing 5 mm of Beard Parker agar (BP) for *S. aureus* and M-17 agar for *S. agalactiae*. One hundred µL of each pathogen (10^7 cfu/mL) were spread in agar plates (spread-plating) having a 17 mm diameter hole made with a sterile punch. Subsequently, cylinders of sterile MRS agar (5 mm in depth and 17 mm in diameter) were inoculated with 0.06 mL of B or B+M and deposited in the holes made in the Petri plates. The plates were incubated at 32ºC for 24 hrs. After incubation, the halos resulting from bacterial growth inhibition were measured. The tests of antimicrobial activity were conducted in duplicate for each of the fractions and for each of the pathogens.

To assess the effect of the substrate (MRS *vs*. MYEG) and storage conditions (lyophilization *vs.* refrigeration) on the antimicrobial activity of *W. confusa* strain and metabolites against the two pathogens, a 2^3 x 7 factorial experiment in a completely randomized design with two replicates was used. The factors were: (1) fermentation substrate with two levels (MRS and MYEG), (2) method of conservation with two levels (refrigerated and lyophilized), (3) biological antimicrobial substance with two levels (biomass and biomass-metabolite), and (4) storage time with seven levels (0, 1, 2, 3, 4, 5, and 6 weeks). To assess the effect of the substrate (MRS *vs.* MYEG) and storage conditions (lyophilization *vs.* refrigeration) on the viability of *W. confusa* strain; a 2^2 x 7 factorial experiment in a completely randomized design was used. The factors were (1) fermentation substrate with two levels (MRS and MYEG), (2) method of conservation with two levels (refrigerated and lyophilized), and (3) storage time with seven levels (0, 1, 2, 3, 4, 5, and 6 weeks). In both cases, analysis of variance was done using SAS version 9.13 (SAS Institute, Inc., Cary, NC). When treatment effects were significant ($p < 0.05$), multiple mean comparisons were done using the Tukey test.

RESULTS AND DISCUSSION

Figure 1 shows the viability of *W. confusa* strain grown in MRS and MYEG fermentation substrates and preserved by refrigeration and lyophilization during six weeks. Significant interaction effect ($p < 0.05$) was found among factors fermentation substrate, method of conservation and storage time, suggesting that the effect of each factor on the viability of *W. confusa* strain depended on the levels of the other two factors. After 4 hrs of fermentation, cell concentration was higher in strain grown in MRS (2.1 x 10^{10} cfu/mL) than MYEG (1.4 x 10^9 cfu/mL). The survival of *W. confusa* strain was significantly reduced due to lyophilization, with final counts immediately after lyophilization of 3.3 x 10^9 cfu/mL for strain grown in MRS and 5.4 x 10^7 cfu/mL for strain grown in MYEG. No further significant loss in cell viability was observed up to the 5th week of storage of lyophilized strain grown in MRS.

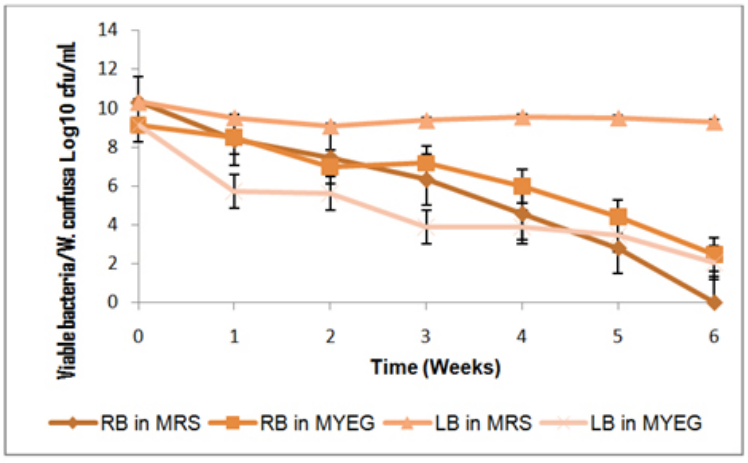

Fig. 1 Viability of *W. confusa* strain (Log cfu/mL) grown in MRS and MYEG (milk-yeast extract-glucose) broth and preserved by refrigeration or lyophilization. RB: refrigerated biomass; LB: lyophilized biomass. The bars indicate standard errors of means.

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The method of conservation significantly affected ($p < 0.05$) the viability of *W. confusa* strain grown in MRS and MYEG substrates. The survival of refrigerated *W. confusa* strain grown in MRS declined progressively until the 6th week of storage. In the case of lyophilized strain, the viability remained essentially unchanged during the study period (six weeks), with an average of 1.9 x 10^{9} cfu/mL. A very different pattern was found for the case of strain grown in MYEG substrate, where strain preserved by lyophilization exhibited less viability than strain preserved by refrigeration.

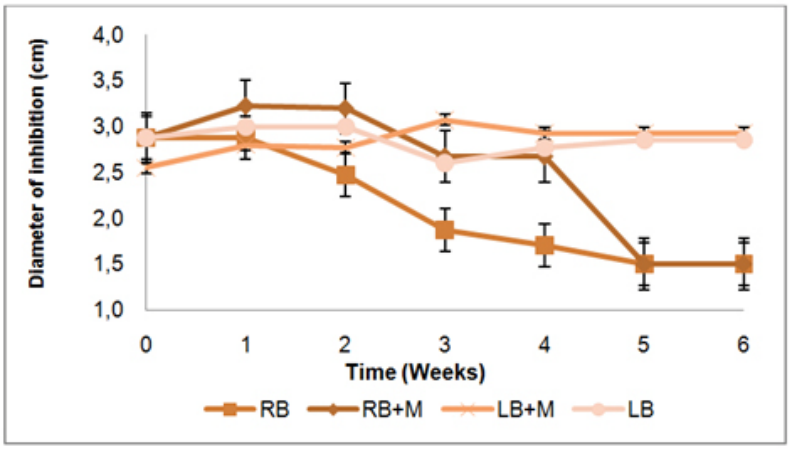

Fig. 2 Antimicrobial activity (zone of inhibition) of *W. confusa* strain and metabolites against *S. aureus*. Strain grown in MRS broth. RB: refrigerated biomass; RB+M: refrigerated biomass and metabolites; LB: lyophilized biomass; LB+M: lyophilized biomass and metabolites. The bars indicate standard errors of means.

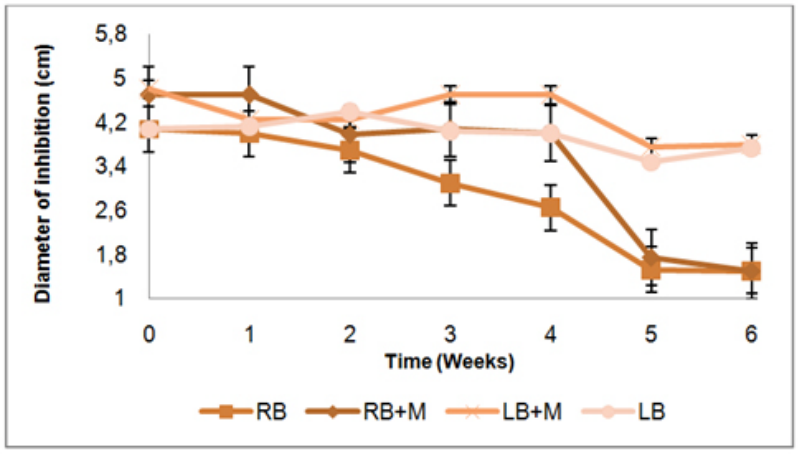

Fig. 3 Antimicrobial activity (zone of inhibition) of *W. confusa* strain and metabolites against *S. agalactiae*. Strain grown in MRS broth. RB: refrigerated biomass; RB+M: refrigerated biomass and metabolites; LB: lyophilized biomass; LB+M: lyophilized biomass and metabolites. The bars indicate standard errors of means.

The observed reduction in viable strain after lyophilization was consistent with results by Zarate and Nader-Macias (2006), who reported a 0.05 to 2 reduction in viable Log cfu/mL after the lyophilization of *L. acidophilus*, *L. paracasei*, and *L. salivarius*. These authors reported that the viability in lyophilized strain stored in lactose or skim milk, strongly declined during the first months of storage. According to Carvalho et al. (2004), the different growth kinetics corresponding to different fermentation substrates, are due to the degree of stress affecting the cell membrane fatty acid composition. The loss of viability

during the lyophilization process has been also linked to physical changes in the cell membrane (Koch et al. 2008).

A significant interaction effect ($p < 0.05$) was found among factors fermentation substrate, conservation method, and storage time on the antimicrobial properties of B and B+M against the two pathogens tested.

Figures 2 and Figure 3 show the inhibitory activity against *S. aureus* and *S. agalactiae* of *W. confusa* strain grown in MRS broth and preserved for up to six weeks by lyophilization or refrigeration. In the case of strain grown in MRS, significant differences due to method of conservation (lyophilization *vs.* refrigeration) were also found. The refrigerated RB+M fraction exhibited a drop in antimicrobial activity after the 2th week with reductions in the diameter of zone of inhibition from 3.20 to 2.68 cm for *S. aureus*, and 5.03 to 3.98 cm for *S. agalactiae* and at the 4th week with reductions from 2.68 to 1.50 cm for *S. aureus* and 3.98 to 1.75 cm for *S. agalactiae*. The refrigerated RB fraction showed reduction in antimicrobial activity after the 1st week of storage. After five weeks of storage, no significant differences were found between RB+M and RB fractions, with average diameters of zone of inhibition of 1.50 for *S. aureus* and 1.53 cm for *S. agalactiae*.

The fractions LB and LB+M showed stable antimicrobial activity through storage time. This pattern was consistent with results observed in the stability study, where lyophilized strain remained viable during storage with 2.1×10^{10} initial cfu/mL and 1.98×10^{9} final cfu/mL after five weeks. On the other hand, a gradual reduction in the number of viable strain was observed in refrigerated samples, which in turn accounted for a loss of inhibitory activity against the pathogens. Lyophilization was effective in maintaining the viability and antimicrobial stability of *W. confusa* strain grown in MRS.

Figure 4 and Figure 5 show the inhibitory activity of *W. confusa* strain grown in the MYEG substrate and stored under refrigeration or lyophilization for six weeks. For strain grown in MYEG, the method of conservation (refrigeration *vs.* lyophilization) showed no significant effect on the antimicrobial properties of strain and metabolites against *S. agalactiae* ($p = 0.4698$) or *S. aureus* ($p = 0.0514$). Consequently, the inhibitory capacity of *W. confusa* strain and its metabolites could be maintained when preserved under both, lyophilization or refrigeration conditions.

These results also suggest that the strain and its metabolites found protective components in milk. Milk contains micro-nutrients, vitamins and minerals that lactic acid bacteria require to grow, and also protects native structures during storage. Tomás et al. (2003) reported that the milk-yeast extract combination proved the most appropriate media for maintaining the viability of *Lactobacillus* strains during storage at -70ºC. According to Carvalho et al. (2004) *L. bulgaricus* better survives storage when grown in a medium containing fructose, lactose and mannose *vs.* glucose only. Substrates containing multiple sugars would produce strain with physiological and morphological characteristics allowing resistance to preservation-induced stress.

In lyophilized samples, the best antimicrobial response against the two pathogens was produced by the LB+M fraction in both, MRS and MYEG substrates. The preservation of the antimicrobial activity was the result of the combined effect of viable bacterial strain and its fermentation products, since lyophilization preserves the native structures of strain and metabolites.

It has been reported that the production of metabolites differs depending on the composition of the growth media. Hofvendahl and Hahn-Hägerdal (2000) reported that homofermentative organisms growing in media containing sugars other than glucose produced minor protective compounds such as intracellular mannitol. Zalán et al. (2010) assessed the production of organic acids by 10 *Lactobacillus* strains in different media (MRS broth and skim milk) and found that all the strains grown in MRS (except *L. plantarum* 01) produced lactic, acetic, butyric, formic, citric, succinic and glutamic acid, while those grown in skim milk produced formic and succinic acid. On the other hand, Otero et al. (2007) reported that *L. gasseri* CRL1412 showed better preservation when suspended in skim milk, because lactose promoted the growth and survival of lactic acid bacteria (Meng et al. 2008). In addition, milk proteins can prevent cell damage by stabilizing membrane constituents and calcium can increase cell survival (Castro et al. 1995; Meng et al. 2008). Soto et al. (2009) studying the viability of *Lactobacillus casei* DSPV 318T, *L. salivarius* DSPV 315T and *Pediococcus acidilactici* DSPV stored in MRS substrate and milk, found that milk was the best substrate to maintain the viability of the three lactic acid bacteria in both refrigeration and lyophilization conditions.

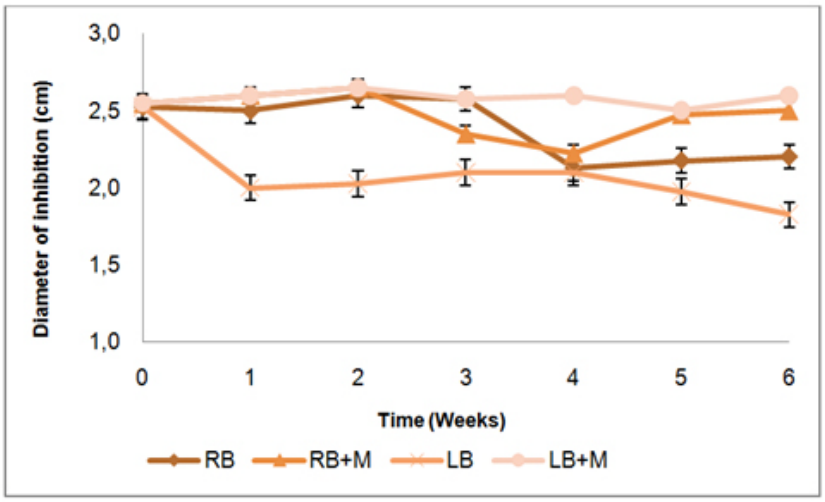

Fig. 4 Antimicrobial activity (zone of inhibition) of *W. confusa* strain and metabolites against *S. aureus*. Strain grown in milk-yeast extract-glucose (MYEG) broth. RB: refrigerated biomass; RB+M: refrigerated biomass and metabolites; LB: lyophilized biomass; LB+M: lyophilized biomass and metabolites. The bars indicate standard errors of means.

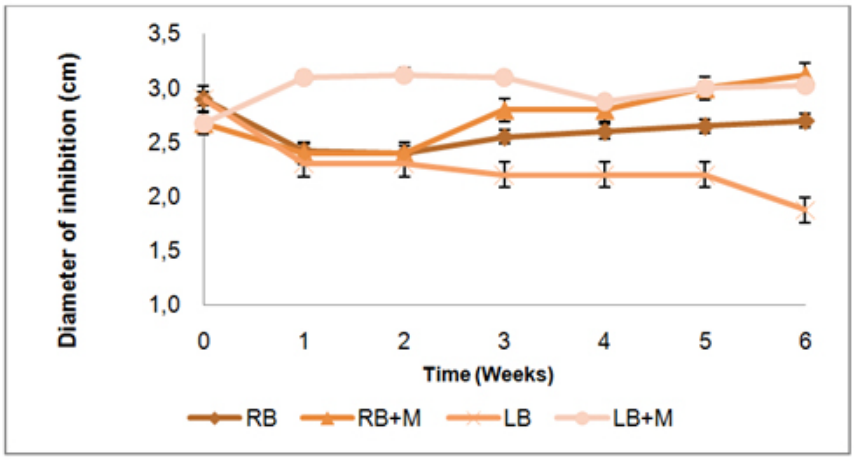

Fig. 5 Antimicrobial activity (zone of inhibition) of *W. confusa* strain and metabolites against *S. agalactiae*. Strain grown in milk-yeast extract-glucose (MYEG) broth. RB: refrigerated biomass; RB+M: refrigerated biomass and metabolites; LB: lyophilized biomass; LB+M: lyophilized biomass and metabolites. The bars indicate standard errors of means.

CONCLUDING REMARKS

W. confusa strain grown in MRS and then lyophilized retained viability and antimicrobial activity after six weeks of storage. In the case of strain grown in milk-yeast extract-glucose, refrigeration was suitable to retain adequate viability and antimicrobial activity for six weeks. Whereas lyophilization and commercial substrates are expensive, our results indicate that it is possible to retain viability and antimicrobial activity of *W. confusa* against mastitis-causing microorganism by using milk-based low cost substrates and refrigeration. Results from this research facilitate the commercial use of this lactic acid bacterium for the prevention and/or treatment of bovine mastitis.

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