ORIGINAL ARTICLE

Effects of plant extracts on the growth of beneficial indigenous lactic acid bacteria (BLAB) for their potential use in preventing bovine reproductive tract infections

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ABSTRACT. There is a renewed interest in products based on phytocompounds, prebiotics and probiotics applied to different hosts to exert effects of immunomodulation, anti-inflammation and analgesia. The microbiome of the bovine reproductive tract can become unbalanced for many reasons, favoring the entry and proliferation of pathogenic microorganisms, currently treated with antibiotics that exert adverse effects and generate antimicrobial resistance. To deal with this situation, "phytobiotic" formulas are proposed that combine phytocompounds and probiotics. This work aims to study the effect of plant extracts, prebiotics and vitamins on the growth of native beneficial lactic acid bacteria (BLAB), to be further potentially applied in the design of phytobiotic formulas. Nine beneficial strains isolated from different bovine ecosystems were evaluated against nine phytocompounds, two prebiotics and five vitamins. Affinity was assayed using the diffusion technique on agar plates, and the effect of the phytocompounds on the growth of lactic acid bacteria by microplates. The growth of all the strains was affected by some plant extracts, showing a stimulating or inhibitory effect. Diffusion-agar plates show that only vitamin A affected the viability of *Lactobacillus johnsonii* CRL1702 at concentrations higher than 7.5 mg/ml. When studying the growth kinetics of the strains with the phytocompounds, the results show that the effect was different in each of one the associated strains + plant extracts, indicating a strain-specific effect of plant extracts on each BLAB strain. Lapacho and Malva stimulate the growth of most microorganisms, then were selected to be combined with BLAB to design a phytobiotic formula with potential therapeutic activity to treat bovine reproductive infections. Plant extracts at the evaluated concentrations did not inhibit the growth of most of the pathogens responsible for endometritis. On the other hand, the highest concentrations of phenolic compounds were detected in Echinacea, Lapacho and Llantén; and the best percentages of antioxidant activity were evidenced in Garlic, Blueberry and Chamomile (<60%).

Keywords: Probiotic lactic bacteria, *Handroanthus impetiginosus*, *Malva sylvestris*, phytobiotics, bovine reproductive health.

INTRODUCTION

The autochthonous microbiota of the different bovine tracts and mucosa are constituted by a wide variety of microorganisms in ecological balance, called "microbiome", which is currently considered one more organ since it provides metabolic activities, coding capacities and fulfills a fundamental role and variety of physiological functions including immunomodulation and prevention of infection in humans and animals (Li *et al*. 2013; Stumpf *et al*. 2013). Lactic acid bacteria (LAB) are one of the most significantly important groups of bacteria in the food industry. They have long been consumed in dairy products all around the world and most of them are classified as "Generally Recognized As Safe" (GRAS) and into Qualified Presumption of Safety (QPS) microorganisms, because they are non-pathogenic, suitable for technological and industrial processes, having the ability to produce many antimicrobial substances (Reuben *et al*., 2020; Shehata *et al*., 2016). In the last decade, LAB has received increased attention and are widely used as probiotics, defined as "live microorganisms that exert health benefits on the host when ingested in adequate amounts" (FAO/WHO, 2006). Furthermore, lactobacilli are also under development as delivery systems for vaccines and treatments (Sun *et al*., 2015).

On the other hand, there is a growing interest in natural ingredients, including plant sources and derivatives, both from consumers and producers in the food and pharmaceutical industries. People search into the market for products that are artificial and synthetic additives-free to promote their health and animal sanity (Marchesi *et al.*, 2020). In almost all countries, plants have been widely used throughout history for the treatment and prevention of different diseases and infections in humans and domestic animals. Today, these traditional treatments are recommended in human and veterinary medicine due to their promising therapeutic efficacy with minimal side effects and reduction of chemotherapeutic drug residues in animal products consumed by humans (Gurib-Fakim, 2006). Most of them are included in the Pharmacopoeia regulations, while the perspective and future approaches of ethnopharmacological research are developed in parallel with advances in clinical and laboratory sciences, mainly phytochemistry and pharmacology (González-Stuart *et al*., 2017). Medicinal plants with their well-established history are an excellent resource of natural products used as an alternative therapy (Mushtaq *et al*., 2018). The limitation of therapeutic options for emerging multi-resistant microorganisms and the urgent need for new (or old uses)

natural and safe combinations are emerging. Then, the concept of phytobiotics was conceived, to combine safe phytoderivatives and probiotic bacteria for the design of new formulations, to advance in the combination/synergy of the immunomodulatory, anti-inflammatory, anticancer, analgesic and antioxidant effects reported individually in both, LAB probiotics and plant extracts (Nader Macías *et al*., 2008; Ayrle *et al*., 2016; Martínez & Lujan, 2011). These bioactive ingredients must be included in formulas in such a way that they must be compatible, protect against harsh environmental and process conditions, and be safely delivered to cells and target organs. In the area of probiotics, our research group has a long history of experiments carried out *in vitro* or in animal models, complemented by studies on the design of different types of vaginal probiotic formulas (Ocaña *et al*., 1999; De Gregorio *et al*., 2016; Leccese Terraf *et al*., 2014).

Therefore, the objective of this work was to evaluate the effect of phytoextracts (powdered) on the growth of bovine homologous vaginal probiotic lactobacilli and their affinity/compatibility to select those to be combined for the further design of a phytobiotic formulas. Also, to determine the phytocompounds effect on bovine vaginal pathogens, and the phenolic content and antioxidant activity of the plant extracts, to define if are related with the beneficial effect. The best combinations were defined to advance in the design of an intravaginal formula to be further applied to the reproductive health of postpartum cows.

MATERIALS AND METHODS

BENEFICIAL AUTOCHTHONOUS LACTIC ACID BACTERIA (BLAB) AND GROWTH CONDITIONS

The bacterial strains used in this study were beneficial lactobacilli previously isolated from three bovine ecosystems: the vaginal tract and mammary gland of cows and the gastrointestinal tract of calves (Tucumán, Argentina). The strains used are included in Table 1, together with the previously determined beneficial properties (Otero *et al*., 2006; Espeche *et al*., 2012; Maldonado *et al*., 2012; Miranda *et al*., 2020). The microorganisms were preserved in yeast extract milk (13% skim milk, 0.5% yeast extract and 1% glucose) at -20 °C, subcultured 3 times in MRS broth (De Man, Rogosa and Sharpe, Biokar Diagnostics, Beauvais, France) and incubated for 12 hours at 37 °C. Protocols were performed with the third subculture at 2%. The vaginal tract strains were isolated from vaginal brushing samples (Otero *et al.,* 2006), from the mammary gland/milk (Espeche *et al*., 2012) adult cows. Calves LAB were obtained from saliva and gastrointestinal tract (faeces) (Maldonado *et al*., 2012).

PREPARATION OF PLANT EXTRACTS, VITAMINS AND PREBIOTICS

The solid vegetable compounds evaluated were obtained from SAPORITI (Buenos Aires, Argentina) and selected

Table 1. Beneficial characteristics of the lactic acid bacteria strains selected and evaluated.

Microorganism	Beneficial properties[•]	Isolation Origin
Lactobacillus gasseri CRL 1412	H_2O_2 producer, hydrophobic, biofilm former and EPS producer	
Lactobacillus gasseri CRL 1421	H_2O_2 and lactic acid producer, hydrophobic, biofilm-forming	
Lactobacillus gasseri CRL 1460	Hydrophobic	Bovine vagine*
Lactobacillus gasseri CRL 1461	Lactic acid producer. Hydrophobic, self-aggregating, high adhesion capacity	
Lactococcus lactis subsp. *lactis* CRL 1656	H_2O_2 and lactic acid producer. Inhibits *S. aureus*, *S. uberis*, *S.agalactiae* and SCN	Bovine mammary gland[γ]
Pediococcus pentosaceus CRL 1831	H_2O_2 producer, biofilm former, EPS +	
Weisella cibaria CRL 1833	Hydrophobic, biofilm-forming, EPS +	
Lactobacillus johnsonii CRL 1693	Lactic acid producer. Self-aggregating, hydrophobic, inhibits *E.coli*, *S. aureus*, *S. dublin*.	
Ligilactobacillus murinus CRL1695	H_2O_2 and lactic acid producer. Self-aggregating, biofilm-forming, inhibits *S. typhimurium*	Faecal matter calves[+]
Limosilactobacillus mucosae CRL1696	H_2O_2 producer	
Ligilactobacillus salivarius CRL1702	Lactic acid producer. Hydrophobic, inhibits *S. typhimurium*	

CRL: Centro de Referencia para Lactobacillus
*Published previously by Otero *et al.* (2006).
γPublished previously by Espeche *et al.* (2012).
+Published previously by Maldonado *et al.* (2012).
•Published previously by Miranda *et al*. (2020).

from the list of natural extracts approved by different Pharmacopoeias (Argentinian Pharmacopoeia; European Pharmacopoeia) for human and veterinary applications, included in Table 2 with their ethnopharmacological properties: *Allium sativa* (Garlic), *Vaccinium myrtillus* (Blueberry), *Atropa belladonna* (Belladonna), *Echinacea angustifolia* (Echinaceae), *Matricaria recutita* (Chamomile), *Handroanthus impetiginosus* (Lapacho), *Malva sylvestris* (Malva), *Larrea divaricata* (Jarilla) and *Plantago major* (Llantén).

COMPATIBILITY OF BLAB WITH PLANT EXTRACTS BY THE AGAR PLATE DIFFUSION TECHNIQUE

The Minimal Inhibitory Concentration (MIC) of the plant extract was determined by the agar plate diffusion technique, by using a range between 1.8 and 30 mg/ml.

They were prepared at 60 mg for the microplate assay, dissolved in 1 ml of 25% alcohol, and stored at 4 °C protected from light until use. The vitamins used were: A, B5, B7 and B12 (ICN Biomedicals Inc, Ohio, USA), while powdered corn syrup (Arcor, Lules, Tucuman, Argentina) and inulin (Sigma-Aldrich, USA) was used as prebiotic and dissolved in water (30 mg/ml).

MRS (De Man, Rogosa and Sharpe) agar plates (Biokar Diagnostics, Beauvais, France) (1% agar) were prepared by inoculating 100 µl of different BLAB ($1x10^6$ and $1x10^8$ CFU/ml) added to the melted and cooled agar. Once solidified, 25 µl (per spot) of the seriated dilutions of plant extracts, vitamins and prebiotics were inoculated, maintained quiescent until complete diffusion on the agar, and incubated at 37 °C for 48 hours. The inhibition zones (in millimeters) were determined by using a manual caliper after. Assays were performed in triplicate.

Table 2. Application/Effect of vegetal extracts (approved in pharmacopoeias) assayed

Scientific name	Vulgar name	Application[*]	References	Pharmacopoeia
Allium sativum	Garlic	Modulation of the immune system and inflammation. GI tract: antidiarrheal Antibacterial - Anti-inflammatory Insect Bites - Injuries and Wounds	Ayrle *et al.*, 2016; Mirabeau 2012; Harris, 2001; Pittler, 2007; Iciek, 2009; Oosthuizen, 2017; Anwar 2017; Cheng, 2018	Argentina 8th edition
Malva sylvestris	Malva	Modulation of the immune system and inflammation - Intestinal colic - Wounds and injuries. Mastitis - Intestinal colic - Eye lesions - Antioxidant	Ayrle *et al.*, 2016; Martínez & Lujan, 2011; Bensaad, 2016; Prudente, 2017; Martins, 2017; Lasparetto, 2011; Vahabi, 2019	Argentina 1er annex
Larrea divaricata	Jarilla	Bumps or swelling - Muscle pain - Placental retention	Martínez & Lujan, 2011; Canale, 2018; Davicino, 2007; Palacio, 2008, 2012; Davicino, 2008; Martino, 2010, 2011; Pedernera, 2006	Argentina 8th edition
Plantago major	Llanten	Shock/inflammation - Wounds and injuries - Antiulcer - Anti-inflammatory - Antidiarrheal - Antitumor.	Martínez & Lujan, 2011; Amos, 2017; Najafian, 2018; Chiang, 2003a, b; Huang, 2009	Argentina 8th edition
Matricaria recitita	Chamomile	Wounds and injuries - Treatment of castration - Antioxidant - Anticancer - Placental retention - Insect and animal bites	Martínez & Lujan, 2011; Miraj, 2016; McKay, 2006; Sharifi-rad, 2018; Park, 2017; Al-Dabbagh, 2019; Mohsenzadeh, 2011	Argentina 8th edition
Echinacea angustifolia	Echinacea	Immune system strengthens and enhancer	Martínez & Lujan, 2011; Barnes, 2005; Arland, 2016; Sharifi-rad, 2018; Sharma, 2009; Stevenson, 2005	European annex 2014
Vaccinium myrtillus	Blueberry	Antioxidants - Anti-inflammatory - Anti-hypertensive - Antimicrobial - Anticancer	Jakesevic, 2013; Korus, 2014; Mykkanen, 2014; Nohynek, 2006; Veljković, 2016	European 8th edition / Argentine Food Code
Atropa belladonna	Belladonna	Antipyretic - Modulates inflammation	Ahmad, 2018; Pedalino, 2004	Argentina 8th edition
Handroanthu simpetiginosus	Lapacho	Anti hyper uricemic - Anti-inflammatory - Antioxidant - Anti-tumor - Antiulcerogenic - Analgesic - Anti-allergy	Ferraz-Filha, 2016; Grazziotin, 1992; Kim, 2012; Suo, 2012	Argentina 3rd annex

[*]Ethnopharmacological effect of plant extractsΩ

GROWTH KINETICS OF BLAB WITH PHYTOCOMPOUNDS BY MICROPLATE TECHNIQUE

The behavior of 9 plant extracts (SAPORITI drugstore, Buenos Aires-Argentina) at a concentration of 60 mg/ml on 10 different BLAB strains was evaluated: *Lactobacillus gasseri* CRL1412, *Lactobacillus gasseri* CRL1421, *Lactobacillus gasseri* CRL1460, *Lactobacillus gasseri* CRL1461, *Lactococcus lactis* subsp. *lactis* CRL1656, *Pediococcus pentosaceus* CRL1831, *Weisella cibaria* CRL1833, *Lactobacillus johnsonii* CRL1693, *Ligilactobacillus murinus* CRL1695, *Limosilactobacillus mucosae* CRL1696 and *Ligilactobacillus salivarius* CRL1702. The solid plant extracts were weighed and resuspended in 1 ml of 25% ethanol and filtered through sterile 0.22μm membrane filters (Biofil-Syringe Filter). The BLAB inoculum was prepared from the third MRS broth subculture with O.D.560nm 0.9-1.0 (10^7-10^8 CFU / ml).

The effect of the plant extracts on the growth of the BLAB strains was evaluated in polystyrene microplates (Extragen-96-well ELISA plate), to which 150 μl of MRS broth (De Man, Rogosa and Sharpe) (Biokar Diagnostics, Beauvais, France) inoculated with 2% BLAB was added. Then 50 μl of the plant extracts were added, gently mixed and incubated at 37 °C for 48 hours. Bacterial growth was determined by optical density (O.D.$_{560nm}$) (Spectronic 20, Bausch and Lomb, Rochester, NY) at 4, 6, 22, 24, 30 and 48 hours. Solvents, individual plant extracts and bacteria in MRS were included as a control. The values of O.D. of bacterial growth were obtained subtracting the O.D. value obtained for each plant extract. All experiments were performed in triplicate.

EFFECT OF PLANT EXTRACTS ON THE GROWTH OF BOVINE REPRODUCTIVE TRACT PATHOGENS

The effect of 9 plant derivatives against different pathogens isolated from the bovine reproductive tract was evaluated by using the modified agar plate diffusion technique (De Gregorio *et al*., 2019). *Escherichia coli* 99/14, *Pseudomonas aeruginosa*, *Streptococcus bovis*, *Enterococcus faecalis* and *Listeria monocytogenes* (Nader *et al*., 2008) were cultured in LAPTg broth (Biokar Diagnostics, Beauvais, France) for 12 hours at 37 °C under microaerophilic conditions, in an anaerobic jar (AnaeroGen Oxoid). Briefly, LAPTg agar plates (15 ml of LAPTg with 1% agar) were inoculated with 1 x 10^6 and 1 x 10^8 CFU/ml of each pathogen (Pasteris *et al*., 2011). Aliquots (25 μl) of the different solid plant extracts dissolved in 25% hydroalcoholic solution (30, 15, 7.5 and 3.25 mg/ml) were added to the holes (4mm in diameter) aseptically made in the agar plates (in triplicate), incubated later for 24 hours at 37°C. The inhibition halos were quantified in mm, determining the Minimum Inhibitory Concentration (MIC) of each of the plant extracts. The extracts were added also as spots on the surface of the pathogens-agar plates (De Gregorio *et al*., 2019).

DETERMINATION OF TOTAL PHENOLIC COMPOUNDS OF PLANT EXTRACTS

The total phenolic content of the phytocompounds was determined by the Zielinski & Kozlowska (2000) technique, with the following modifications: 100 μl of each plant extract (1mg/ml) and 900 μl bidistilled water were mixed with 100 μl Folin-Ciocalteu reagent (Sigma-Aldrich, Darmstadt, Germany) (diluted to 50% in bidistilled water). After 2 minutes at room temperature, 400μl of a Na_2CO_3 solution (Ciccarelli, Santa Fe, Argentina) (15.9%, in bidistilled water) was added and left to stand for 10 minutes in the dark. Then 200 μl were seeded in microplates (in triplicate). Absorbance was measured in a microplate reader at 765 nm in a UV-VIS spectrophotometer (Spectronic 20, Bausch and Lomb, Rochester, NY). The calibration curve was prepared with gallic acid (GA) (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in ethanol as a standard, in a concentration range of 2.5 to 1000 μg. The regression equation used was y = 0.0395x + 0.1633, and the correlation coefficient R2 = 0.9907. The results were expressed in mg of GAE extract/ml.

ANTIOXIDANT ACTIVITY OF PLANT EXTRACTS

The antiradical activity was evaluated by the photometric method, according to Wu *et al*. (2003). The DPPHu radical (1,1-diphenyl-2-picryl hydrazyl, Sigma-Aldrich, Darmstadt, Germany) was dissolved in 80% methanol up to an absorbance equal to 1.0 at 517 nm. 100 μl aliquots of each phytocompounds (1mg/ml) were mixed with 200 μl of DPPHu. The decrease in absorbance was determined in a microplate spectrophotometer (Spectronic 20, Bausch and Lomb, Rochester, NY) at 517 nm, with readings every 30 seconds for 10 minutes. The percentage of inhibition of DPPHu radical was calculated through the equation described by Burda & Oleszek (2001).

$$AAR= 100 \times (1-Am/A0)$$

Where Am is the absorbance of the mixture at an infinite time and A0 is the absorbance of the DPPH solution before the addition of the samples.

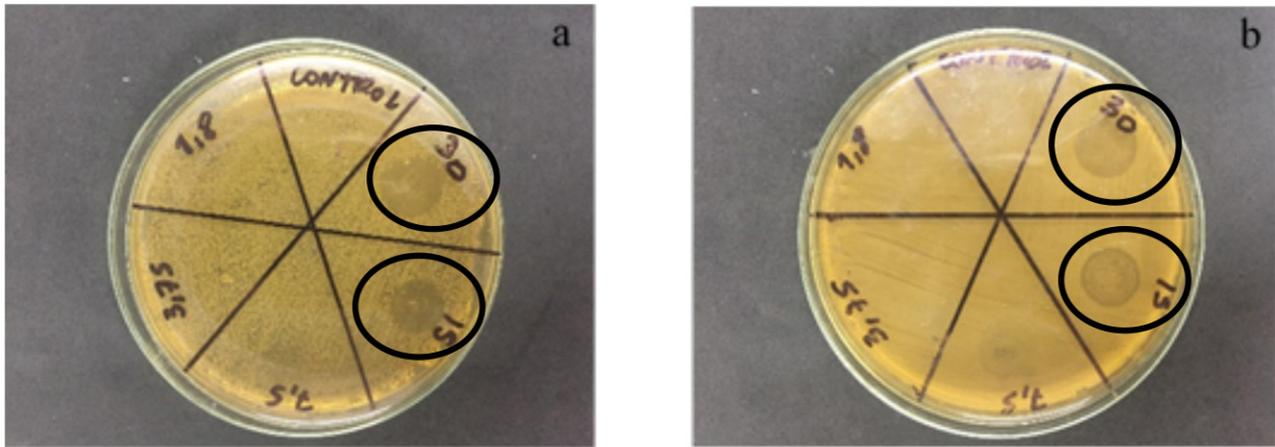## STATISTICAL ANALYSIS

The results were subjected to analysis of variance (ANOVA) and a significance level of 5% (p≤0.05) was applied, using the Minitab analytical program.

RESULTS

BLAB COMPATIBILITY WITH PLANT EXTRACTS

The diffusion method on MRS agar plates determined that all plant extracts assayed are compatible with BLAB, as

Figure 1. Inhibition of vitamin A on *Ligilactobacillus salivarius* CRL1702: 10^6 CFU/ml (a) and 10^8 CFU/ml (b). The black circles indicate the inhibition spots.

well as inulin and corn syrup used as prebiotics, except *L. salivarius* CRL1702 (10^6 and 10^8 CFU/ml) (Figure 1A-B) which was inhibited by vitamin A at 30 and 15 mg/mL concentrations (MIC = 7.5 mg/mL).

BLAB GROWTH IN THE PRESENCE OF DIFFERENT PLANT EXTRACTS

The plant extracts under study affected the growth of many BLAB strains since they were either stimulated or inhibited. Figure 2 shows the effect of different phytocompounds on BLAB growth, indicating that the effect is strain-specific. The claimed pharmacological effects of all the phytoderivatives assayed (Table 2), were not related to the degree of inhibition exerted on the growth of the strains; the growth of each BLAB with the added phytocompounds, evaluated by O.D.560nm, indicated that the effect is different in each one of the strains + plant extracts associations. Some examples are shown in Figure 2: a-g: represents obligate heterofermentative bacteria (OHe) and, h-i: obligate homofermentative lactobacilli (OHo). The growth in standard medium (MRS) of each strain is represented by a dotted line. Figure 2a shows the behavior of *L. gasseri* CRL1412 stimulated by Lapacho, Malva, Llanten and Manzanilla during the first 6 hours, but inhibited in a higher degree by Blueberry, Belladonna, Echinacea and Garlic. However, it is strongly stimulated by Jarilla starting at 12 hours. *L. gasseri* CRL1421 follows a growth pattern similar to CRL1412, showing good growth together with Lapacho and Malva, but inhibited by Belladonna, Llanten and Echinacea (Figure 2b). Figure 2c indicated the growth kinetics of *L. gasseri* CRL1460 strongly stimulated by Lapacho and Malva at 3 and 6 hours, respectively. After 6 hours, this strain was inhibited by most of the plant extracts, except Jarilla. *L. gasseri* CRL1461 (Figure 2d) which showed the highest growth between 3 and 6 hours. Malva, Manzanilla, Lapacho, Llanten and Equinacea produced even higher growth than in the standard broth

(MRS broth). Lapacho, Malva and Llanten facilitated the growth of *L. lactis* subsp. *lactis* CRL1656 during the first 6 hours. Malva reaches a maximum O.D.540nm at 24 hours (1.43 ± 0.039), followed by Echinacea with an $O.D_{540\,nm}$ of 1.33 ± 0.10; while Belladonna suppressed the growth (Figure 2e). Figure 2f shows the growth of *P. pentosaceus* CRL1831 with plant extracts, indicating that Garlic and Malva inhibited this strain, while Blueberry, Lapacho and Manzanilla favor its growth at 24 hours. *L. salivarius* CRL1702 shows similar kinetics but only Llantén allowed higher growth rates at 24 h. Blueberry negatively affects both strains (Figure 2g).

The growth of *W. cibaria* CRL1833 is represented in Figure 2h, where Blueberry, Garlic, Jarilla, Belladonna and Echinacea were showed to inhibit it, while Lapacho and Malva strongly stimulated. *L. mucosae* CRL1696 evidenced an inhibitory pattern of all the plant extracts during the first 6 hours, however, Malva, Llantén, Lapacho and Echinacea produced a higher growth than in MRS at 24 hours (Figure 2i).

The degree of interaction between BLAB and plant extracts is summarized in Figure 4, showing the compatible or non-compatible combinations: stimulating (green) or inhibitory (red) effect of the phytocompounds on the growth of each strain. Gray boxes indicate "No effect" because there is no modification of the growth with the phytoderivatives when compared to the MRS control.

EFFECT OF PLANT EXTRACTS ON THE GROWTH OF UROGENITAL PATHOGENS

The plant extracts assayed at different concentrations was not able to inhibit the growth of most of the frequent pathogenic microorganisms that cause bovine metritis, although the antimicrobial effect of many of them was previously reported (Oosthuizen *et al*., 2017; Martínez & Lujan 2011; Nohynek *et al*., 2006; Sharifi-Rad *et al*., 2018; Canale *et al*., 2018).
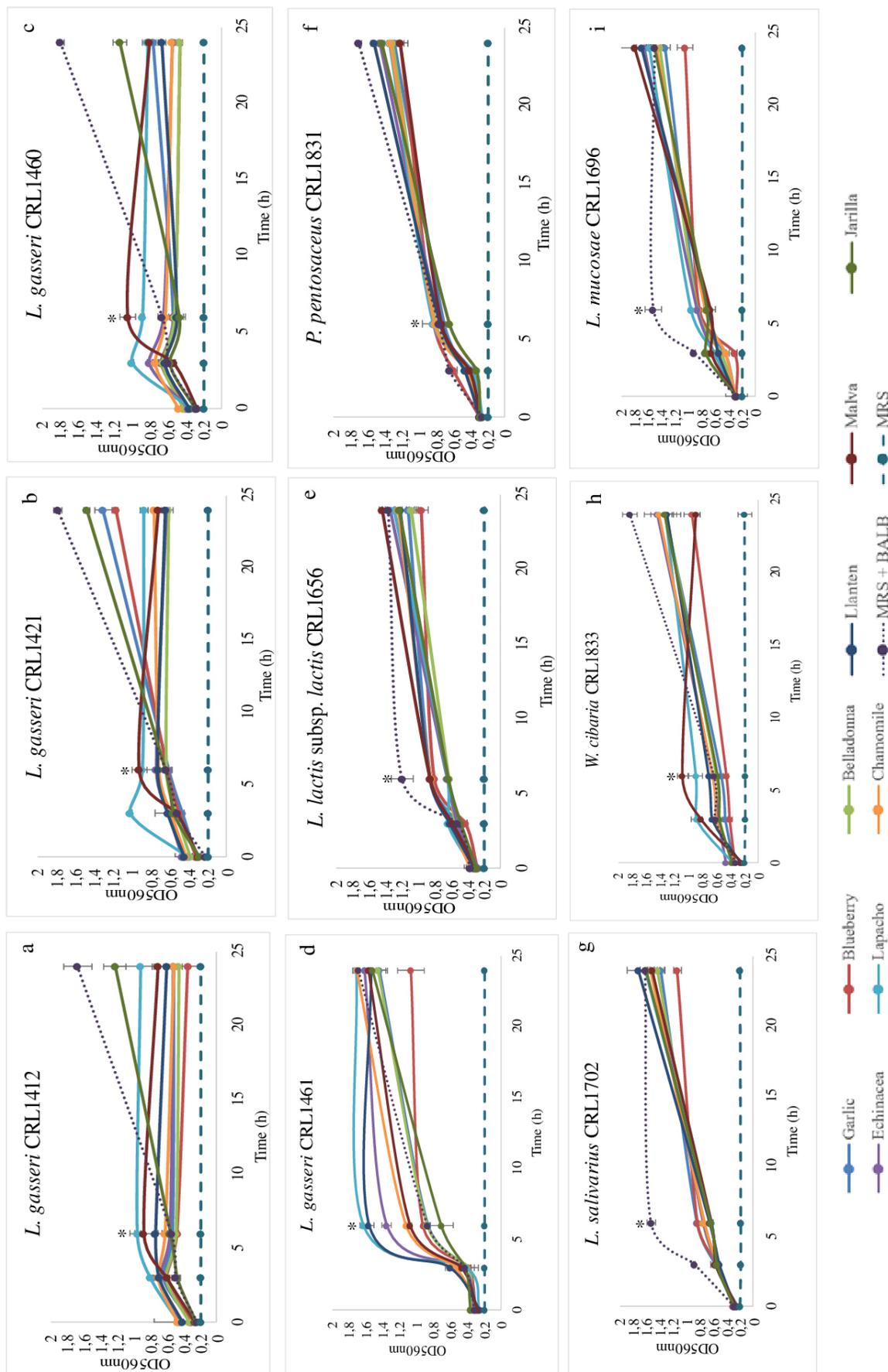
Figure 2. Effect of plant extracts on the growth of obligate heterofermentative (a-g) and obligate homofermentative (h-i) BALB. The asterisk (*) indicates significant differences (p<0.05) in the growth of the strain in different combinations of MRS + plant extract tested according to Tukey's test.

QUANTIFICATION OF TOTAL PHENOLIC COMPOUNDS (TPC) AND ANTIOXIDANT ACTIVITY IN PLANT EXTRACTS

The results obtained by evaluating the content of TPC and the antioxidant activity of the nine plant extracts were determined to select those with higher antioxidant activity, to promote the beneficial effect when combined with probiotics to the phytobiotic formula. Echinacea is the plant extract with the highest concentration of phenolic compounds. Lapacho and Jarilla did not show significant differences between them, or between Garlic and Belladonna. On the other hand, Blueberry and Malva did not present significant differences between them, showing the lowest TPC values (Figure 3a). Garlic, Blueberry, and Chamomile recorded the highest percentages of antioxidant activity, without significant differences between them, followed by Malva. Belladonna was the phytocompound with the lowest antioxidant activity (Figure 3b).

DISCUSSION

There is increasing interest in natural ingredients, including plant sources and their derivatives, and beneficial microorganisms from both consumers and producers in the food and pharmaceutical industries. People search the market for products and foods free of artificial additives that can promote their health, many of them with a very long application based on concepts or uses derived from custom. The same interest is shown in animal production systems.

On the other hand, the use of lactic acid bacteria as probiotics in different mucosal sites is widely recommended (ISSAP, 2016; McFarland *et al.*, 2018). Specifically, in the bovine reproductive system, different scientists have shown that the application of an adequate combination of probiotics could effectively counteract an endometrial infection by *E. coli* (Genis *et al.*, 2016, 2017) and improve productive parameters (Ametaj *et al.*, 2014). Then, to increase/enhance beneficial effects (such as immunomodulation, and inhibition of pathogenic microorganisms), it is possible to combine them with extracts from plants currently or historically applied to the urogenital tract to design different formulas intended to exert or produce a synergistic effect on the host. Therefore, it is of the utmost importance to determine their compatibility and if the phytoderivatives affect the growth of BLAB to be used in a single beneficial formula.

In human medicine, some results have been published regarding the administration of a vaginal gel containing *Thymus vulgaris* and *Eugenia caryophilus* together with two *Lactobacillus* strains specifically formulated in slow-release capsules for the treatment of recurrent bacterial vaginosis or vulvovaginal candidiasis (Murina *et al.*, 2018) however, there are no previous studies that report the compatibility between the two bioactive components of the commercial formula. It is very important, and as a first step for the design of the formula, to evaluate the effect
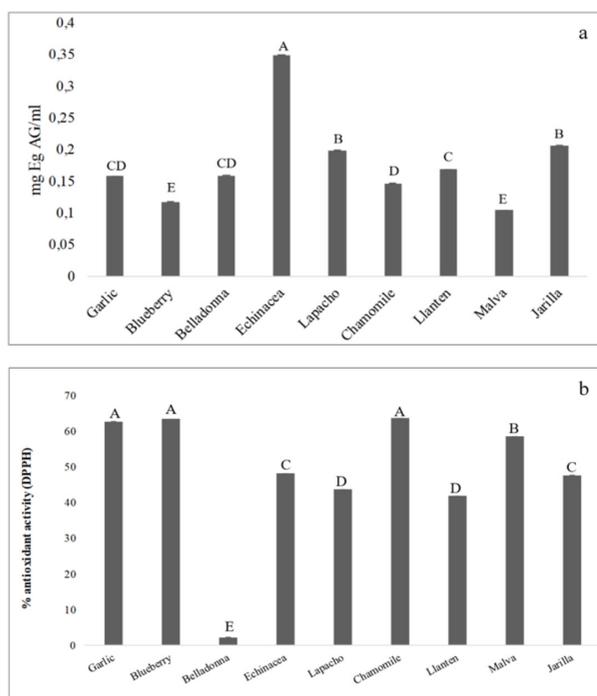

Figure 3. Quantification of phenolic compounds (a) and antioxidant activity (b) in plant extracts.

The different letters represent statistically significant differences (p<0.005) (Tukey's test).

Figure 4. Compatibility between beneficial vaginal lactic acid bacteria in the presence of plant extracts. Red: "Inhibitory effect"; Green: "Stimulating effect"; Shades of color indicate greater or lesser inhibition ( ) or stimulation ( ) and "No effect" ( ).

of the phytoderivatives on the growth of lactobacilli. The results of this work show the interactions of a long list of phytoderivatives approved in the Pharmacopoeias to be used for both oral and local administration with bovine BLAB probiotic strains characterized in our laboratory. The compatibility of phytoderivatives and strains of bovine lactic acid bacteria in veterinary medicine has not been previously studied, therefore, the issue is of the utmost importance, and considered as a first step to design a novel formula, to evaluate the effect of phytoderivatives, whether liquid or extract, on the growth of BLAB. The results obtained in this work indicate that the effect of the phytoextracts is dependent on the strain since there are no general rules. Each strain and each extract must be tested to define their optimal or adequate combinations. In this work, the evaluation of their combinations was carried out through MIC tests and growth kinetics, which allowed defining which of them could be adequately combined. Different publications have reported the individual use of lactic acid bacteria and plant extracts as curative and/or preventive treatments in veterinary medicine (Pellegrino *et al*., 2017; Maldonado *et al*., 2017; Mansilla *et al*., 2020; Ayrle *et al*., 2016; Martínez & Lujan 2011; Lillehoj*et al*., 2018; Marume *et al*., 2017). Phytotherapy represents one of the most widely applied non-conventional medicines in both human and veterinary medicine (Hahn *et al*., 2005). According to Viegi *et al*. (2003), large animals (cattle, horses, sheep, goats and pigs) represent 70.5% of domestic animals treated with herbal remedies, because phytotherapy is mainly used on organic farms to further reduce the use of allopathic medicines. Although the use of phytotherapeutic products is increasing in animals, there are few studies and clinical trials reported in the literature on the therapeutic use of different plants for reproductive disorders in cows (El-Shanawani, 1996; Bruni *et al*., 1997; Lans *et al*., 2000; Uncini Manganelli *et al*., 2001; Alawa *et al*., 2002; Guarrera, 2005).

As previously indicated, there are no available studies on the combination of phytoderivatives and probiotic lactic acid bacteria and their application in the bovine reproductive system for the prevention of infections, reason by which there is a limitation to discuss/comparing the results obtained in this work with some other scientific reports. For this reason, the beneficial effects of the probiotic microorganisms and the plant extracts assayed in this work are included individually. Supported by the limited information available and the constant search for natural and innocuous products, the combination of probiotics and phytocompounds for veterinary use is an interesting and promising vacancy to study, even though there is a requirement to further assay their combination in the host animal.

The plant extracts evaluated in this work were selected for their variety of pharmacological effects and their use in veterinary medicine. Lapacho (*Handroanthus impetiginosus*) was the plant extract that stimulated the growth of the largest number of BLAB strains evaluated,

with statistically significant differences (at 6 hours). Lapacho is traditionally used in folk medicine and mentioned for its therapeutic effects and included in pharmaceutical forms such as infusions and tinctures, mainly as an analgesic, anti-inflammatory and antitumor agent (Grazziotinv *et al*., 1992). Suo *et al*. (2012) demonstrated in an *in vitro* assay that compounds isolated from Lapacho bark significantly suppressed IL-1β and TNF-α cytokine production without significant cytotoxicity in LPS-stimulated cells, suggesting that Lapacho suppresses production the inflammatory cytokines. Therefore, the combination of Lapacho + BLAB strains is a promising strategy for cattle.

Another phytoderivative, Malva (*Malva sylvestris)*, shows a stimulating/promoting effect on *L. gasseri* CRL1412, *L. gasseri* CRL1421*, L. gasseri* CRL1460 and *W. cibaria* CRL1833. Several studies report the use of *Malva sylvestris* for veterinary purposes. The decoctions of whole plants are administered to cattle to treat colic, the leaves applied in compresses have shown high effectiveness in the treatment of mastitis in cattle, infusions and decoctions of aerial flowery parts were used against inflammation, wound infection, diarrhea in calves, its use as a curative for skin, reproductive and nervous disorders was also reported (Gasparetto *et al*., 2011; Viegi *et al*., 2003). Martins *et al*. (2017) evaluated the anti-inflammatory activity of the Malva extract and observed an inhibition of the release of proinflammatory mediators. Then the association of Malva with selected BLAB strains results in another interesting combination.

Plantain (*Plantago major*) was able to promote the growth of *L. gasseri CRL1461, P. pentosaceus CRL1831, L. mucosae CRL1696* and *L. salivarius CRL1702*. Velasco *et al*. (2006) found that the antibacterial activities of Plantago major leaves and seeds were shown to inhibit *Escherichia coli, Bacillus subtilis* and *Candida albicans* cultures. The rupture of the cell walls of Gram-positive bacteria and the formation of vesicles in Gram-negative bacteria (Metiner *et al*., 2012) were observed by electron microscope evaluations.

Jarilla (*Larrea divorcata*) shows a stimulating effect on the growth of vaginal strains, which is interesting, since different publications have shown that extracts of *L. divaricata* have immunomodulatory properties (Davicino *et al*., 2007, 2010). Jarilla also exhibited *in vitro* antimicrobial effects against pathogens (Quiroga *et al*., 2004; Stege *et al*., 2006). Then it is possible to indicate that the behavior of stimulation or inhibition of the extract is directly related and depends on each strain and species of microorganism. The Jarilla is an autochthonous phytoderivative widely used in northern Argentina.

Garlic and Belladonna extracts inhibited the growth, to different degrees, of all strains tested. Although their important therapeutic effects have been studied, it is not possible to combine them with probiotic microorganisms, possibly due to their antimicrobial properties.

Combinations of beneficial lactic acid bacteria and plant extracts for veterinary therapeutic purposes have not been previously reported. Phytobiotic formulations for female vaginal application were studied by combining probiotic lactobacilli with different active ingredients (Marchesi *et al*., 2020), but no products are marketed in our country. These authors advanced in the selection of viable beneficial lactobacilli for their inclusion in the design of phytobiotic formulas for the health of the female urogenital tract, based on their compatibility with natural compounds with ethnopharmacological properties. The phytocompounds studied in this work did not show to inhibit the growth of pathogenic microorganisms responsible for urogenital infections in cows, even though their antimicrobial effects were reported in the literature. Even though this is not an encouraging result, the definitive effect must be assayed in the host animal, because the *in vitro* experiments, predictive in some aspects, sometimes do not agree with the *in vivo* behavior or clinical/animal studies.

The concentration of phenolic compounds and the antioxidant activity in the extract are not directly correlated with the effect on the growth of BLAB nor with the compatibility between them. Further studies should be carried out to determine if any specific type of component is responsible for the stimulating or inhibitory effect. Lillehoj *et al*. (2018) reported that the beneficial effects of phytoextracts are attributed to their antimicrobial and antioxidant properties, which is not demonstrated in our results. The inclusion of phytoderivatives in the diets of animals should alter and stabilize the intestinal microbiota and thus reduce toxic microbial metabolites in the intestine, due to their direct antimicrobial properties on various pathogenic bacteria, resulting in the alleviation of intestinal challenges and immune stress, which would lead to improved performance (Kim *et al*., 2015). In ruminants, tannins, included in phenolic compounds, modulate the ruminal and intestinal microbiota and improve the growth of certain bacterial populations. The effects of tannins on the rumen microbiota may be different depending on the molecular nature of these polyphenols (Vasta *et al*., 2008; Min *et al*., 2015).

CONCLUSION

The BLAB strains that showed an affinity with the phytoextracts were: *L. gasseri* CRL1421, *L. gasseri* CRL1460, *L. gasseri* CRL1461 and *W. cibaria* CRL1833 with Lapacho, Malva, Llanten and Jarilla, which can be used to design phytobiotic formulas due to their pharmacological effects (antibacterial, anti-inflammatory, immunomodulatory and healing). These results are original, because the compatibility of natural extracts with probiotic lactic acid bacteria strains was studied, in a way to combine the beneficial effect of the two bioactive compounds in the host, which should be further assayed in *in vivo* protocols.

COMPETING INTERESTS STATEMENT

ETHICS STATEMENT

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