

# Effect of phytoderivatives on the growth of homologous beneficial vaginal lactobacilli (BVL) strains and their compatibility for the design of phytobiotics for the vaginal tract health

**Antonella Marchesi**

Consejo Nacional de Investigaciones Cientificas y Tecnicas

**Jessica Salva**

Consejo Nacional de Investigaciones Cientificas y Tecnicas

**Cecilia Aristimuño Ficoseco**

Consejo Nacional de Investigaciones Cientificas y Tecnicas

**Birgitt Wiese**

Medizinische Hochschule Hannover

**Maria Elena Fatima Nader-Macias** (✉ [fnader@cerela.org.ar](mailto:fnader@cerela.org.ar))

CERELA-CONICET <https://orcid.org/0000-0001-7526-1860>

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## Research

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# Abstract

**Background:** Lactobacilli are the predominant bacteria in the vaginal tract, and while they are present, can prevent infectious situations, being used as probiotics for the health of the tract. Phytoderivatives are used historically to prevent or maintain the health of the tract, and is possible to combine both bioactives for novel vaginal formulas.

The aim of this work was to study the effect of phytoderivatives (individual or combined extracts) on the growth of homologous vaginal lactic acid bacteria and their compatibility, to go further in the design of a combined pharmabiotic formula for the prevention or treatment of urogenital tract infections.

**Methods:** the effect of phytochemicals approved by Pharmacopoeias was evaluated on the growth of beneficial lactic acid bacteria previously isolated from the vaginal tract. Statistical methods, as CART, were applied to determine the stimulatory or inhibitory effect of the vegetal sources on bacterial growth by applying different methodology. Compatibility between phyto and lactobacilli was determined, and the phenolic compounds contents quantified, to define the optimal conditions for the formula design.

**Results:** The BVL strains showing highest growth values with most of the phytoderivatives under study were *L. gasseri* CRL 1361, *L. ga.* CRL 1509 and *L. ga.* CRL 1263, and *L. jensenii* CRL 1333 and *L. jen.* CRL 1317, indicating the behavior is strain-dependent, and vegetable-compound-affected. The most adequate phytoderivatives were: *Carica*, *Centella*, *Plantago*, *Uva ursi\**, *Zarzaparrilla\**, *Cola de caballo*, *Echinaceae*, *Ortiga* and *Palo Azul*.

**Conclusions:** The BVL strains showing optimal resistance to the phytochemicals and compatibility with them were *L. gasseri* CRL 1320, 1307, 1509, *L. salivarius* CRL 1296 and *L. rhamnosus* CRL 1332 combined with *Uva ursi\**, *Zarzaparrilla\** and *Echinaceae* that will be used for the design of optimal phytobiotic formulas.

## Introduction

The new concept of microbiome on the human and animal mucosa demonstrated during the last decade and the high variety of related functions described, supports the application of probiotic formulations designed with host and tract homologous microorganisms. Lactobacilli are a diverse group of more than 200 different species that occupy diverse nutrient-rich niches associated with humans, animals, plants and food [1]. They are used widely in biotechnology and food preservation, and are being explored as therapeutics [2]. Lactobacilli and other Generally Regarded as Safe (GRAS) and Qualified Presumption of Safety (QPS) genera are widely used as probiotics, which can be administered to help the host in the restoration of the indigenous microbiota, stimulate the immune system, or either protect against the income of pathogenic microorganisms. On the other side, there is an ever-growing interest on natural ingredients, including vegetal sources, derivatives and formula, both by consumers and producers in the food and pharma industries. In fact, people are looking for those products in the market which are free from artificial and synthetic additives and can promote their health, many of them with a very long

application supported by concepts or custom-derived uses. In the ethnopharmacological area, the knowledge related to the traditional uses of medicinal plants is totally in the custody of elder community members and local herbalists [3]. A long list of them is included in the Pharmacopeia resources, indicating they can be used safely for different situations [4, 5]. Historically, they were used with different proposals, effects reviewed by different authors [6]. Also, the limitation of the therapeutic options for emerging multidrug resistance microorganisms, and the urgent need of new (or old-uses) natural and safe combinations is emerging [7]. Then, the concept of phytobiotics has been conceived, to combine safe phytoderivatives and probiotic bacteria for the design of new formulations, resulting in the combination/synergy of the two beneficial effects. These bioactive ingredients should be formulated in such a way that they are compatible, protects them against harsh process and environmental conditions and could be delivered safely to the target organs and cells.

On the probiotic area, our research group has a long history of experiments performed “in vitro” or in animal models, complemented by studies on the design of different type of vaginal probiotic formula [8, 9, 10]. Then, the aim of this work was to study the effect of phytoextracts (macerated or powders) on the growth of homologous vaginal probiotic lactobacilli (by applying different methods) and their compatibility to select those than can be combined for the design of phytobiotic formulas. The growth kinetics and compatibility assays were designed and evaluated through the application of specific statistical models, to select those with an optimal behavior. Also, the phenolic content was evaluated, and the best combination defined to go further in the design of different type of formulas for the reproductive tract health.

## Materials And Methods

### Bacterial strains and growth conditions

The bacterial strains used in this study were beneficial vaginal lactic acid bacteria (BVLAB) (*Lactobacillus reuteri*, *L. gasseri*, *L. rhamnosus*, *L. salivarius*, *L. delbrueckii*, *L. johnsonii*, *L. paracasei*) isolated from women vagina (Tucuman, Argentina). They are included in Table 1, together with their beneficial properties previously determined [8, 9, 10, 11, 12]. Microorganisms were stored in milk-yeast extract (13% non-fat milk, 0.5% yeast extract and 1% glucose) at -20°C [8]. Before the experiment they were subcultured three times, and later grown in MRS broth (BIOKAR, Argentina) for 13 h at 37°C. BVLB were inoculated at 2% for the experimental assays, and incubated for 12 and 24 hours at 37°C. The third subculture was monitored by optical density measurements at 560 nm (O.D.<sub>560nm</sub>) (Spectronic 20, Baush and Lomb, Roscherter, NY), until reaching an O.D. of 0.9- 1.0, corresponding to  $1 \times 10^6 - 1 \times 10^8$  CFU/ml.

### Genetic Identification of lactic acid bacteria.

The method applied to identify the BVL selected by their beneficial characteristics by the application of molecular-related techniques is described in previous works [13]. The strains, as identified by this method, are included in Table 1.

## Vegetal material sources

The natural solid compounds assayed were obtained from SAPORITI (Buenos Aires, Argentina), and selected from the phytoderivate compounds lists approved by the Pharmacopoeia (Argentinean Farmacopea [4]; European Farmacopea[5]) for human applications: *Allium sativa* (Ajo), *Vaccinium myrtillus* (Arandano), *Atropa belladonna* (Belladona), *Peumus boldo* (Boldo), *Hydrocotyle asiática* (Centella), *Echinacea angustifolia* (Echinaceae), *Arctostaphylos Uva-ursi* (Uva ursi), *Matricaria recutita* (Manzanilla), *Smilax aspera* (Zarzaparrilla) and *Lapacho* (Lapacho) [14, 15, 16, 17]. Suspensions of 30, 15 and 7.5 mg dissolved in 1 ml of 40% alcohol were used (obtaining cloudy solutions or suspensions). Liquid natural compounds were obtained from Fitot Laboratory (Tucuman- Argentina) elaborated by maceration: *Aesculus hippocastanum*, *Capsella bursa-pastoris*, *Hydrocotyle asiatica*, *Hamamelis virginiana*, *Caléndula officinalis*, *Matricaria recutita*, *Bacharis articulata*, *Mentha pulegium*, *Equisetum arvenseu*, *Mentha piperita*, *Betula pendula*, *Minthostachys mollis*, *Taraxacum officinale*, *Chelidonium majus*, *Peumus boldus*, *Cynara scolymus*, *Rosmarinus officinalis*, *Fabiana imbricata*, *Amaranthus muricatus*, *Dryopteris filix mas*, *Cuassia amara*, *Dysphania ambrosioides*, *Mentha pulegium*, *Ruta graveolens*, *Cyclolepis genistoides*, *Alternanthera pungens*, *Geoffroea decorticans*, *Achyrocline satuireioides*, *Aloysia polystachya*, *Arctostaphylos uva-ursi*, *Smilax aspera*, *Urtica dioica*). The phytocompounds were used in 40% ethanol as solvent. The extracts, listed in Table 2, were stored at 4°C until use. All of the phytoderivatives under assay were selected by their ethno-pharmacological properties described in Pharmacopoeia and used for human applications. They can be applied either as oral route, external uses (skin surface) or by the two ways (Table 3 and Table 4).

## Growth Kinetics of BVLAB with phytocompounds by Microplate technique

Polystyrene microplates (Extragen microplate -ELISA plate 96 well) wells were filled with the 150 µl of MRS broth (Biokar, Argentina) inoculated with BVL at 2%. Later, 50 µl of each one of the liquid phytocompounds was added to the wells, gently mixed and incubated at 37°C during 24 h. The bacterial growth was determined by optical density (O.D.<sub>560nm</sub>) (Spectronic 20, Baush and Lomb, Roscherter, NY) at 3, 6, 9, 12 and 24 h at 37°C. The growth kinetics were plotted to evaluate the behavior of each one of the strains with the different phytocompounds. Solvents, individual phytocompounds, and strains grown in MRS were included as control. All the experiments were performed by triplicate. The growth kinetics to indicate the effect of the phytocompounds on each strain was plotted, and also used to calculate the percentage of the maximal O.D. in MRS broth, represented in the color table as: *Stimulating effect*: high ("H-S": >70%), medium ("R-S": 70%-35%) or low ("L-S": <35%). *Inhibitory effect*: high ("H-I": >70%), medium ("R-I": 70%-35%) or low ("L-I": <35%). *No significant effect*: "N-E" (No effect).

## Compatibility between microorganisms and phytocompounds by the plate agar diffusion technique

MRS agar plates (Biokar, Argentina) (1% agar) were inoculated with 100 µl of different concentrations of BVL ( $1 \times 10^6$  and  $1 \times 10^7$  CFU/ml). Holes of 4 mm were aseptically prepared in the agar plates, and later inoculated with 25µl of serial dilutions in 40% ethanol (from 30 mg/ml) of each one of the

phytocompounds under study, settled for diffusion, and later incubated in microaerophilic conditions at 37°C. The inhibitory halos were measured after 48 h (in millimeters). Controls included the solvent used for each one of the phytocompounds. The assays were performed for triplicate [18].

### **Quantification of polyphenolic content in natural compounds**

The photometric assay was applied to quantify the phenolic concentration in the phytocompounds under study [19] and the absorbance was determined at 750 nm using a UV-VIS spectrophotometer (Spectronic 20, Baush and Lomb, Roscherter, NY). The calibration curve was obtained with Gallic Acid (GA) (Sigma Aldrich, Argentina) as standard (concentration range 2.5–1000µg) with ethanol as control (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The regression equation was:  $y = 0.0395x + 0.1633$ , and the coefficient of correlation  $R^2 = 0.9907$ . The results were expressed in mg GAE·g<sup>-1</sup> extract. The experiments were performed by triplicate.

### **Statistical evaluation.**

The bacterial growth parameters were estimated applying the 4-parameter modified Gompertz-model:

$$OD = N_0 + A \cdot \exp\{-\exp[(\mu \cdot e/A) \cdot (\lambda - t) + 1]\}$$

where OD is the optical density at time t (time of growth in hours),  $N_0$  the OD at  $t = 0$ , A the difference between the final and the initial ODs,  $\mu$  the maximum specific growth rate ( $h^{-1}$ ),  $\lambda$  the lag phase time in hours and e the base of the neperian logarithm.

For the estimation of the parameters constrained nonlinear regression was performed. This method uses a sequential quadratic programming algorithm. For the parameter standard errors and confidence intervals the method of Bootstrapping was applied, using repeated samples from the original data set. For each growth curve 100 bootstrap samples were taken.

To identify subgroups of combinations of strains with phytocompounds with optimal growth a Classification and Regression Tree (CART) analysis was performed. The CART analysis is capable of detecting complex interactions of variables that might be missed by standard statistical approaches. The estimated growth parameter A was included as independent variable and the parameters strain and phytocompound were included as predictors in the analysis. Only experiments with an estimated parameter A (O.D.) of at least 0.8 and an estimated lag phase shorter than 8 h were selected for the CART analysis.

For the analyses and graphical presentations the statistical programs SPSS 25, S-Plus 8.1 and STATISTICA 12 were used. The CART analysis was performed with Salford Predictive Modeler 8.2. [20].

## **Results**

### **Growth of BVL in presence of different extracts**

The growth of many strains was affected by the phytochemical under assay, because they were either stimulated or inhibited. The complete list of phytochemicals assayed is shown in Table 2. Some examples of the effect of phytochemicals on the BVL growth are indicated in Figure 1 and 2 that means the effect of each one of the vegetal compounds on the different BVL is strain-specific, and not related with the metabolic groups in which they were originally classified. The claimed effects of all the phytochemicals under assay are shown in Table 2 and 3, being not related to the inhibitory effect produced on the BVL growth.

The growth of the strains at 37°C was evaluated through O.D.<sub>560nm</sub> every 3 hours, being different in each one of the associations of the different strains and phytochemicals. Those bacteria that had a higher O.D.<sub>560nm</sub> than 0.8 and less than 8 hours lag phase were chosen to plot in the graphs. Some examples are shown in Figure 1: A- B: represent obligate homofermentative bacteria (OHe); C- D: facultative heterofermentative (FHe), and E-F: to obligate homofermentative lactobacilli (OHo). The growth in standard media (MRS) of each strain of lactobacillus is represented by a dotted line. Figure A and B show the behavior of *L. reuterii* CRL 1324 and *L. reuterii* CRL1327. The strains are stimulated by *Carica* (*Car*) and *Betula* (*Bet\**), and both of them inhibited by *Chelidonium* (*Che\**) and *Echinacea* (*Ech*). But, *L. reuterii* CRL 1327 is highly stimulated by *Carica* (*Car*) and *Betula* (*Bet\**), and more inhibited by *Centella* (*Cen*).

Figures C and D show the growth of *L. rhamnosus* CRL 1511 and *L. rhamnosus* CRL 1332 indicating that the strain CRL 1332 was not affected by *Carica* (*Car*) and *Betula* (*Bet\**), while favored the *L. rhamnosus* CRL 1511 growth.

*Lactobacillus jensenii* CRL 1333 and *Lactobacillus gasserii* CRL 1264 growth were plotted in Fig E and F; *Chelidonium* (*Che\**), *Lippia* (*Lip\**) and *Echinaceae* (*Ech*) affected the growth of the two strains. However *L. jensenii* CRL 1333 growth was slightly inhibited by *Centella* (*Cen*).

In most of the BVL evaluated, *Betula* (*Bet\**) and *Carica* (*Car*) extracts did not show an inhibitory effect on their growth.

Related to the optimal concentration of vegetal extracts to be used, Figure 2 shows that different concentration of *Allium sativa* (Fig 2B, 2C, 2D), *Hamamelis* (Fig 2E, 2F, 2G) and *Uva ursi* (Fig 2H, 2I, 2J) exert an inhibitory effect on the BVLAB growth related with the increased concentration of the phytoextracts added, being higher when adding 3 mg/ml than 0.75 mg/ml. Further studies are required to determine if the strains would be able to grow after an induction or adaptation phase, previously to their use for the technological application.

### **Liquid Phytochemicals and BVL: "Classification and regression tree analysis"**

With the experimental data obtained from the combination of all the phytochemicals (Table 2, 3) and lactic acid bacteria strains (Table 1), the growth parameters of each one of the strains associated with each phytochemical was calculated. The cut point established were O.D.: 0.8, lag phase shorter than 8 h, and incubation time 24 hours, with these conditions 184 trials were selected for the analysis. The

statistical evaluation performed through the CART (Classification and regression tree analysis) software, showed the following results:

The selected trials show a mean estimated O.D. of 1.007 growth (Fig. 3). The algorithm then divides the "root node" containing all selected trials into two nodes using the variable "strain", including 128 assays with an average growth of 0.938 O.D. (BVL in the box "Terminal Node 1"), while on the right 56 trials are included with an average growth of 1.163 O.D. ("Node 2"). Node 2 is then divided using the predictor "phytocompound". The resulting "Terminal node 2" includes 29 trials with an average growth of 1.026 O.D., while "Node 3" includes 27 trials showing an average growth of 1.309 O.D.

"Node 3" is then split into two terminal nodes using the predictor "strain": "Terminal Node 3" includes 18 assays with an average growth of 1.182 O.D. and "Terminal node 4" contains 9 trials with an average growth of 1.564 O.D.

Hence the CART analysis identifies four subgroups of trials (terminal nodes) with an average growth ranging from 0.938 O.D. to 1.564 O.D.

### **BVL and phytocompounds compatibility**

The behavior of 24 BVL classified into different metabolic groups were assayed with 25 different natural alcoholic extracts. The degree of interaction between BVL and the vegetal extracts is summarized in Table 5, showing the compatible or non-compatible combinations: stimulatory (green) or inhibitory effect (red) of the phytocompounds on the growth of each strain. The BVL growing with the phytoderivatives is indicated with different colors: green boxes: "High Stimulation", (higher of 70% growth than in control MRS broth); light-green color "Medium Stimulation" (between 70% and 35% growth higher than control) and "Low Stimulation" (35% higher growth than the control). At the same time, the red boxes indicated "High Inhibition" (70% decrease of the maximal growth compared with control), while orange boxes represent "Medium Inhibition" (those strains inhibited between 70% and 35%); pink boxes "Low Inhibition" (BVL inhibited lower than 35%); The yellow boxes, "No Effect", did not modify their growth with phytoderivatives when compared with control MRS.

The results summarized in Table 5 indicate that behavior or growth of each one of the BVL strain under evaluation was different depending on the phytocompound added to the assay. Some of the phytoderivatives showed a similar behavior when added to BVL, as for example: "**Medium Stimulation**": *Carica papaya* (Ca), *Yerba meona* (Yme), *Echinacea* (Ech), *Zarzaparrilla* (Zar), *Palo azul* (Paz) and *Ortiga* (Ort). "**Medium Inhibition**" was produced by *Lippia* (Lip\*), *Perilla* (Pe), *Zarzaparrilla* (Zar\*) and *Hamamelis* (Ham\*), while "**No effect**" was detected with *Zarzaparrilla* (Zar), *Uva ursi* (Uva) and *Palo azul* (Paz).

The BVL strains showing highest growth, indicating their compatibility with most of the phytoderivatives under study were *L. gasseri* CRL 1361, *L. gasseri* CRL 1509 and *L. gasseri* CRL 1263; *L. jensenii* CRL 1333,

*L. jensenii* CRL 1317. The strains strongly inhibited by most of the phytochemicals were *L. fermentum* CRL 1287 and *L. salivarius* CRL 1296.

*Uva ursi* (Uva) and *Zarzaparrilla* (Zar) did not affect ("N-E") the growth for *L. reuteri* CRL 1327, *L. reuteri* CRL 1324 and *L. rhamnosus* CRL 1511.

However, *Yerba meona* (Yme) showed a High Stimulating ("H-S") effect against strains classified into different metabolic groups such as: *L. gasseri* CRL 1263, *L. gasseri* CRL 1320 and *L. gasseri* CRL 1261. But, some strains were affected by *Yerba meona* (Yme) with a Medium Stimulating ("R-S") effect. *L. reuteri* CRL 1324, *L. salivarius* CRL 1328, and *L. reuteri* CRL 1327, *L. rhamnosus* CRL 1332 and *L. mucosae* CRL 1508 had a Low Stimulation (L-S) compared with the control.

*L. gasseri* CRL 1261 showed a better results, because the growth was favored by most of the phytochemicals evaluated (supplementary material).

The pure extract of *Yerba meona* (Yme), *Palo azul* (Paz) and *Echinaceae* (Ech), as well as the mixtures corresponding to *Carica* (Car), *Calendula* (Cal) and *Betula* (Bet\*) did not significantly affect the growth of lactic acid bacteria.

### **Phenolic content in the phytochemicals**

The phenolic compounds were quantified trying to define if they are related with their beneficial effect or compatibility with BVL. All the phytoextracts showed to contain phenolic compounds in different quantities, showed in Fig 4, where eleven extracts with content higher than 500 µg eq Galic acid/ml are indicated. *Hamamelis* showed the higher content of phenolics. *Manzanilla* and *Zarzaparrilla* did not show significant differences among them, as well as *Echinaceae*, *Chelidonia* and *Zarzaparrilla*\*. *Echinaceae* and *Yerba meona* did not show significant differences between them. *Betula* and *Perilla* did not have a significant difference, however, *Plantago* presented a significant difference with *Betula*, but not with *Perilla*.

## **Discussion**

The use of phytochemicals is generally transmitted as a consequence of their ancient applications, most of them lately rediscovered, or under stronger evaluation. Scientists have published some experimental data related with isolated vegetal derivatives, for example on specific infections of urogenital pathogens, as *Candida* vaginal infections [21], some viruses [22], and parasites as *Trichomonas* [23] or bacteria [24]. In addition, they are widely recommended and included in the design of vegetal-derived formulas for postmenopausal women or for reproductive disorders [25].

It is widely known that *Uva ursi* and *Ortiga* have shown antibacterial properties against different microorganisms [26] showing results that were expected. But *Hamamelis* and *Aesculus* that are natural derivatives used by their vascular properties, mainly to improve the venous circulation, have shown some

results that were not expected. Many of them were used for the inhibition of pathogens related with the tract, as shown in Table 2 and 3.

On the other side, the use of Lactic acid bacteria as probiotics at different mucosal sites is widely recommended [27, 28]. Then, is possible to combine those phytoderivatives currently or historically applied to the urogenital tract with beneficial lactic acid bacteria with probiotic properties in a way to design different formula directed to exert or produce a synergistic effect on the host. In this way, is of main importance to determine if the phytoderivatives affect the BVL growth and if they are compatible to be used in a unique beneficial formula. The results of this work shows the interactions of a long list of phytoderivatives approved in the Pharmacopeias to be used either for oral or local administration with probiotics BVL characterized in our laboratory. There is no many data on the compatibility of phytoderivatives and vaginal lactobacilli strains published. The paper of Murina [29] reports the administration of a vaginal gel containing *Thymus vulgaris* and *Eugenia caryophyllus* in conjunction with two *Lactobacillus* strains specifically formulated in slow-release capsules in treating bacterial vaginosis, or recurrent vulvovaginal candidiasis disease, recommended their use in the acute treatment. But there is no previous studies reporting the compatibility between the two bioactives constituents of the commercial formula. Then, is of main importance, and as the first step for the formula design, to evaluate the effect of the phytoderivatives, either as liquids or extracts on the BVL growth. The results obtained in this work indicate that the effect is phytoderivative and strain dependent, because there is no general rules. Each strain and each extract must be assayed in a way to define their optimal or adequate combinations. The classification of lactobacilli into metabolic groups supported by their sugar-catabolic ways, either as homofermentative or heterofermentative, does not predict or indicate any type of compatibility, resistance or behavior when combined with the wide variety of vegetal derivatives under assay. The evaluation of their combinations was performed in this work through the growth kinetics, and by MIC assays, allowing to define which of them could be combined adequately.

The very high number of experimental protocols applied to determine the compatibility of the two bioactives under study did not allowed to define and decide promptly the optimal or most adequate combination. Then, the use of statistical models, and the CART analysis is a widely applied tool to define the most adequate combinations, when a huge number of analytical or experimental data are available [30]. The application of statistical methods, and CART analysis, have contribute to go further in the decision and selection of the most adequate BVL strains and phytoderivatives in a way to combine them in a phytobiotic formula. The results indicate that the most adequate phytoderivatives to be combined with BVL are *Carica*, *Centella*, *Plantago*, *Uva ursi*\*, *Zarzaparrilla*\*, *Cola de Caballo*, *Echinaceae*, *Ortiga* and *Palo azul*.

The concentration of the phenolic compounds on the extract is not directly correlated with the effect on the BVL growth or in the compatibility between them. Further studies must be performed to determine if some specific type of components is responsible of the stimulatory or inhibitory effect.

## Conclusion

The BVL strains showing compatibility with the phytoextracts were *L. gasseri* CRL 1320, 1307, 1509, *L. salivarius* CRL 1296 and *L. rhamnosus* CRL 1332 with *Uva ursi*\*, *Zarzaparrilla*\* and *Echinaceae*, which were selected supported by their ethnopharmacological effects (antibacterial, anti-inflammatory and healing) for the further design of phytobiotic formula

## Declarations

**Ethics statements:** not applicable.

**Data statements:** the data included in the manuscripts are original, and had not been published previously. They were not submitted to other scientific editorial. All the authors agree on the data and results included in the manuscript.

**Availability of data and materials:** please contact author for data request.

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**Competing interest:** The authors declare that they have no competing interests.

### Authors' contributions

1. Experimental procedures. References acquisition. Draft of results.

**JAS.** Experimental procedures. Draft of results

**CAF.** Genetic identification of the strains

1. Validation. Statistical evaluation.

**MEFN:** Conceptualization; Formal analysis; Funding acquisition; Investigation; Methodology; Supervision; Writing - original draft; review & editing.

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## Tables

Table 1. *Lactobacillus* strains used in this work and their main probiotic characteristic.

Metabolic group	<i>Lactobacillus</i> strains	Beneficial characteristics	References
Obligate homofermentative	<i>L. gasseri</i> CRL1252	H <sub>2</sub> O <sub>2</sub> production, high hydrophobicity, pathogens inhibition	Ocaña et al. 1999; Juárez Tomas et al. 2011; De Gregorio et al. 2019
	<i>L. gasseri</i> CRL1255	H <sub>2</sub> O <sub>2</sub> and lactic acid production, pathogens inhibition	Ocaña et al. 1999; Juárez Tomas et al. 2011; De Gregorio et al. 2019
	<i>L. gasseri</i> CRL1256	H <sub>2</sub> O <sub>2</sub> and lactic acid production, pathogens inhibition	Ocaña et al. 1999; Juárez Tomas et al. 2011; De Gregorio et al. 2019
	<i>L. gasseri</i> CRL1261	H <sub>2</sub> O <sub>2</sub> production, high hydrophobicity	Ocaña et al. 1999; De Gregorio et al. 2019
	<i>L. gasseri</i> CRL1263	H <sub>2</sub> O <sub>2</sub> and lactic acid production, high hydrophobicity, pathogens inhibition, colonization of BALB/c mice vaginal tract, adhesion to fibrinogen and mucin	Ocaña et al. 1999; De Gregorio et al. 2019
	<i>L. gasseri</i> CRL1264	H <sub>2</sub> O <sub>2</sub> and lactic acid production	Ocaña et al. 1999; De Gregorio et al. 2019
	<i>L. gasseri</i> CRL1265	H <sub>2</sub> O <sub>2</sub> and lactic acid production, high hydrophobicity, pathogens inhibition	Ocaña et al. 1999; Juárez Tomas et al. 2011; De Gregorio et al. 2019
	<i>L. gasseri</i> CRL1268	H <sub>2</sub> O <sub>2</sub> production, pathogens inhibition	Ocaña et al. 1999; Juárez Tomas et al. 2011; De Gregorio et al. 2019
	<i>L. gasseri</i> CRL1270	H <sub>2</sub> O <sub>2</sub> production, pathogens inhibition, selfaggregation	Ocaña et al. 1999; Juárez Tomas et al. 2005, 2011; De Gregorio et al. 2019
	<i>L. gasseri</i> CRL1290	H <sub>2</sub> O <sub>2</sub> production	Ocaña et al. 1999; De Gregorio et al. 2019
	<i>L. gasseri</i> CRL1307	H <sub>2</sub> O <sub>2</sub> and lactic acid production, high hydrophobicity, pathogens inhibition	Ocaña et al. 1999; Juárez Tomas et al. 2011;

			De Gregorio et al. 2019
	<i>L. gasseri</i> CRL1311	H <sub>2</sub> O <sub>2</sub> production, high hydrophobicity	Ocaña et al. 1999; De Gregorio et al. 2019
	<i>L. gasseri</i> CRL1314	H <sub>2</sub> O <sub>2</sub> production	Ocaña et al. 1999; De Gregorio et al. 2019
	<i>L. gasseri</i> CRL1320	H <sub>2</sub> O <sub>2</sub> and lactic acid production, high hydrophobicity, pathogens inhibition, biofilm formation	Ocaña et al. 1999; Leccese Terraf et al. 2012; De Gregorio et al. 2019
	<i>L. gasseri</i> CRL1322	H <sub>2</sub> O <sub>2</sub> production	Ocaña et al. 1999; De Gregorio et al. 2019
	<i>L. gasseri</i> CRL1509	H <sub>2</sub> O <sub>2</sub> and lactic acid production, high hydrophobicity, selfaggregation, pathogens inhibition, colonization of BALB/c mice vaginal tract, adhesion to fibrinogen and mucin	Ocaña et al. 1999; Juárez Tomas et al. 2011; De Gregorio et al. 2019
	<i>L. jensenii</i> CRL1313	H <sub>2</sub> O <sub>2</sub> production, high hydrophobicity	Ocaña et al. 1999; De Gregorio et al. 2019
	<i>L. jensenii</i> CRL1317	High hydrophobicity	Ocaña et al. 1999
	<i>L. jensenii</i> CRL1333	H <sub>2</sub> O <sub>2</sub> production, high hydrophobicity	Ocaña et al. 1999; De Gregorio et al. 2019
	<i>L. jensenii</i> CRL1349	H <sub>2</sub> O <sub>2</sub> production	Ocaña et al. 1999; De Gregorio et al. 2019
	<i>L. johnsonii</i> CRL1292	H <sub>2</sub> O <sub>2</sub> production	Ocaña et al. 1999; De Gregorio et al. 2019
	<i>L. salivarius</i> CRL1296	H <sub>2</sub> O <sub>2</sub> production	Ocaña et al. 1999; De Gregorio et al. 2019
	<i>L. salivarius</i> CRL1328	bacteriocin production	Ocaña et al. 1999; Vera Pingitore et al. 2009
Facultative heterofermentative	<i>L. paracasei</i> CRL1512	H <sub>2</sub> O <sub>2</sub> production	Ocaña et al. 1999; De Gregorio et al. 2019
	<i>L. rhamnosus</i> CRL1332	H <sub>2</sub> O <sub>2</sub> production, high hydrophobicity, biofilm formation, high resistance to lyophilization, pathogens inhibition, colonization of BALB/c mice vaginal tract, adhesion to fibrinogen and mucin	Ocaña et al. 1999; Juárez Tomas et al. 2005, 2011; Leccese Terraf et al. 2012; De Gregorio et al. 2019

	<i>L. rhamnosus</i> CRL1511	H <sub>2</sub> O <sub>2</sub> production, high hydrophobicity, biofilm formation, adhesion to fibrinogen and mucin	Ocaña et al. 1999; Leccese Terraf et al. 2012; De Gregorio et al. 2019
Obligate heterofermentative	<i>L. fermentum</i> CRL1287	H <sub>2</sub> O <sub>2</sub> production, high hydrophobicity	Ocaña et al. 1999; De Gregorio et al. 2019
	<i>L. reuteri</i> CRL1324	H <sub>2</sub> O <sub>2</sub> and lactic acid production, high hydrophobicity, biofilm formation, pathogens inhibition, colonization of BALB/c vaginal tract mice, adhesion to fibrinogen and mucin	Ocaña et al. 1999; Leccese Terraf et al. 2012, 2016; De Gregorio et al. 2015, 2019
	<i>L. reuteri</i> CRL1327	H <sub>2</sub> O <sub>2</sub> production, selfaggregation, biofilm formation	Ocaña et al. 1999; Leccese Terraf et al. 2012; De Gregorio et al. 2019
	<i>L. mucosae</i> CRL1508	H <sub>2</sub> O <sub>2</sub> production, high hydrophobicity	Ocaña et al. 1999; De Gregorio et al. 2019

Table 2. Phytocompounds (pure and combined extracts) assayed in this work

N°	Scientific name	Vulgar name	Abbreviation	Extracts	Ingredients into the mixture
1	<i>Aesculus</i>	AESCULUS	Aes*	C	<i>Castaño de India. Bolsa de pastor. Hamamelis. Manzanilla. Calendula</i>
2	<i>Bacharis</i>	BACHARIS	Bac*	C	<i>Bacharis. Poleo. Manzanilla. Cola de Caballo. Menta</i>
3	<i>Betula</i>	BETULA	Bet*	C	<i>Diente de leon. Palo azul. Peperina menta. Cola de caballo</i>
4	<i>Carica papaya</i>	CARICA	Car	P	
5	<i>Hydrocotyle asiática</i>	CENTELLA	Cen	P	
6	<i>Chelidonium</i>	CHELIDONIUM	Che*	C	<i>Celidonia. Diente de leon. Boldo. Romero</i>
7	<i>Equisetum</i>	EQUISETUM	Equ*	C	<i>Palo pichi. Palo azul. Yerba meona</i>
8	<i>Dryopteris filix mas</i>	FILIX MAS	Fil*	C	<i>Cuassia. Cola de caballo. Paico. Ruda</i>
9	<i>Hamamelis</i>	HAMAMELIS	Ham*	C	<i>Hamamelis. Calendula. Manzanilla</i>
10	<i>Lippia</i>	LIPPIA	Lip*	C	<i>Poleo. Ruda. Palo azul. Yerba de pollo. Chañar</i>
11	<i>Matricaria</i>	MATRICARIA	Mat*	C	<i>Manzanilla. Llanten. Marcela. Burro. Tomillo</i>
12	<i>Perilla frutescens</i>	PERILLA	Per	P	
13	<i>Plantago lanceolata</i>	PLANTAGO	Pla	P	
14	<i>Uva ursi</i>	UVA URSI	Uva*	C	<i>Uva Ursi. Cola de caballo. Hamamelis</i>
15	<i>Zarzaparrilla</i>	ZARZAPARRILLA	Zar*	C	<i>Ortiga. Palo Azul. Cola de caballo</i>
16	<i>Caléndula officinalis</i>	CALENDULA	Cal	P	
17	<i>Aesculus hippocastanum</i>	CASTAÑA DE INDIA	Cas	P	
18	<i>Equisetum arvense</i>	COLA DE CABALLO	Cdc	P	
19	<i>Echinacea purpúrea</i>	ECHINACEA	Ech	P	
20	<i>Matricaria recutita</i>	MANZANILLA	Man	P	
21	<i>Urtica dioica</i>	ORTIGA	Ort	P	
22	<i>Cyclolepis genistoides</i>	PALO AZUL	Paz	P	
23	<i>Amaranthus muricatus</i>	YERBA MEONA	Yme	P	
24	<i>Acrtostaphylos uva-ursi</i>	UVA URSI	Uva	P	
25	<i>Smilax aspera</i>	ZARZAPARRILLA	Zar	P	

Reference: The \* means different phytochemicals extracting into the same solution- "P": Pure extracts (one natural compounds); "C": Combined extracts (mixture of different phytochemicals, which are detailing in the last column)

Table 3. Effects and characteristics of the Liquid Phytochemicals used in this work

SCIENTIFIC NAME	VULGAR NAME	Abbreviation	APPLICATION	USES	PHARMACOPOEIA
<i>Achyrocline satureioides</i>	<i>Marcela</i>	Mar	Digestive effect, antidiabetic, cholagogic, antispasmodic, carminative	oral way	Argentine annexed 2009
<i>Acrtostaphylos uva-ursi</i>	<i>Uva ursi</i>	Uva	Antibacterial, astringent, anti-inflammatory effects in the genitourinary tract; bleaching agent and control of hyperpigmentation disorders of skin.	oral way/ topic use	Argentine/ British Pharmacopeia
<i>Aesculus hippocastanum</i>	<i>Castaña de India</i>	Cas	Improvement the circulation of venous return and the capillary resistance; decreases edema resulting from lymphatic congestion or inflammation.	topic use	Argentine 8th edition
<i>Aloysia polystachya</i>	<i>Te de burro</i>	Bur	Digestive and carminative effect	oral way	Argentine 8th edition/ European
<i>Alternanthera pungens</i>	<i>Yerba del pollo</i>	Ypo	Digestive effect	oral way	Argentine 6th edition
<i>Amaranthus muricatus</i>	<i>Yerba meona</i>	Yme	Diuretic, antitumor, drastic purging, warts, herpes, depurative	oral way	
<i>Atropa belladonna</i>	<i>Belladona</i>	Bel	Irritable bowel syndrome among others	oral way	Argentine 8th edition
<i>Bacharis articulata</i>	<i>Bacharis</i>	Bac	Digestive effect	oral way	Argentine 6th edition
<i>Caléndula officinalis</i>	<i>Caléndula</i>	Cal	Healing and topical anti-inflammatory effect; emulsifier and astringent properties; promote healing of eczema and other forms of inflamed skin (dermatitis); treatment of pruritic or	oral way/ topic use	Argentine 8th edition

			Inflammatory skin conditions; effective for fungal infections of the skin and nails		
<i>Capsella bursa-pastoris</i>	<i>Bolsa de pastor</i>	Bdp	healing effect	topic use	Argentine/ Spanish 4ta edition
<i>Carica papaya</i>	<i>Papaya</i>	Pap	Digestive effect, antifebrile, antihypertensive, anthelmintic, in osteoarthritis and edema. In external application, venous ulcers heal. eczema, erysipelas, psoriasis, freckles and warts	oral way	Mexican
<i>Chelidonium majus</i>	<i>Celidonia</i>	Cel	Choleretic, cholagogic, spasmolytic, mild laxative; anti-inflammatory, antineoplastic, antiviral and antifungal (topical)	oral way/ topic use	European
<i>Chenopodium ambrosioides</i>	<i>Paico</i>	Pai	Eupeptic, diuretic, emenagogo, antiparasitic, febrifuge and carminative effect; skin, digestive cramps, cough and hemorrhoids prevent	oral way	Argentine annexed 2009
<i>Cuassia amara</i>	<i>Cuasia</i>	Cua	Analgesic and prevents formation of blood clots	topic use	Argentine 4th edition
<i>Cyclolepis genistoides</i>	<i>Palo azul</i>	Paz	Antiinflammatory activity.	oral way	
<i>Dryopteris filix mas</i>	<i>Helecho macho</i>	Hma	Antiparasitic	oral way	Argentine annexed 2009
<i>Echinacea purpúrea</i>	<i>Echinacea</i>	Ech	Bacterial, viral and protozoal infections, including infections of the digestive, respiratory and	oral way/ topic use	European

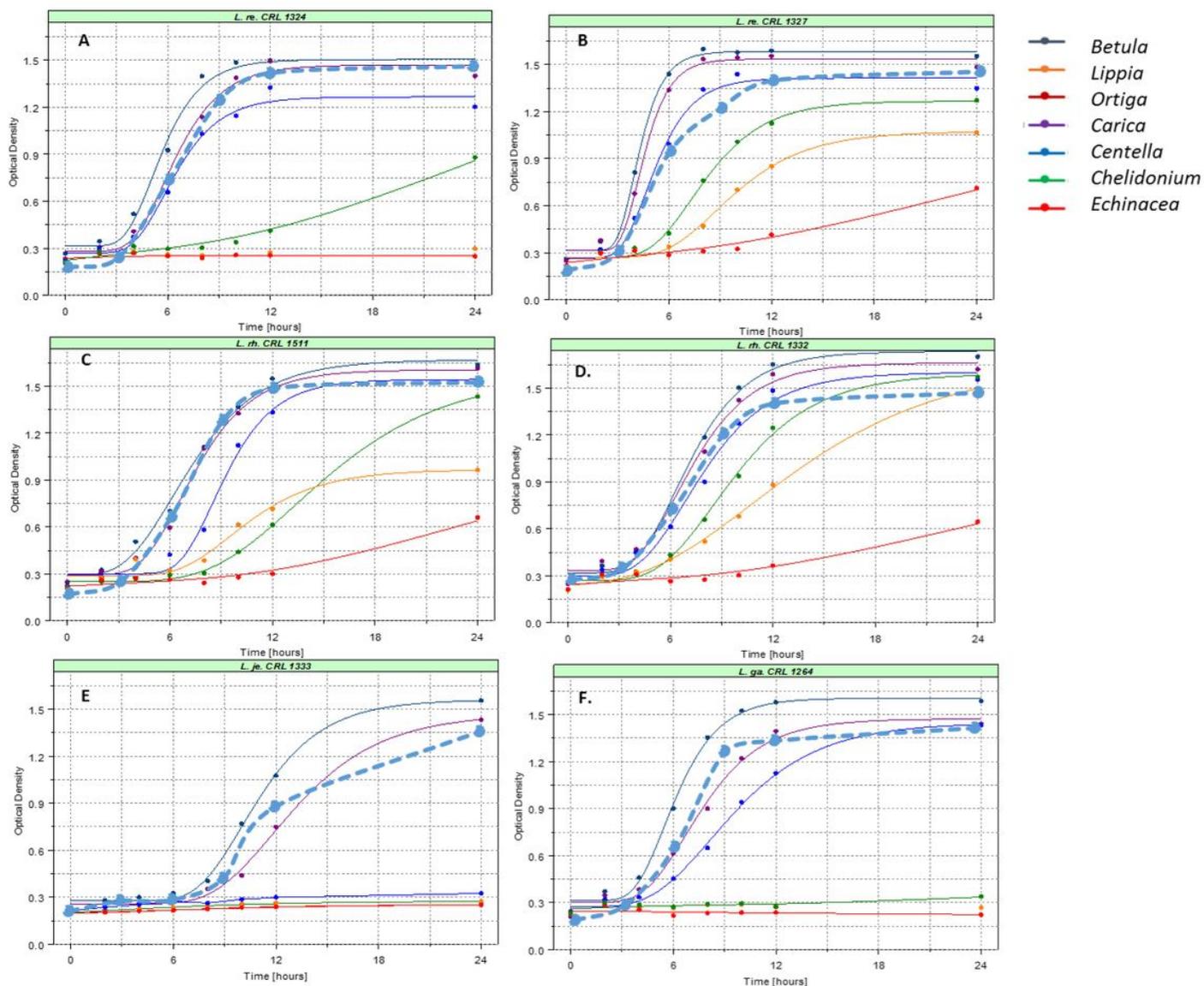
			urinary tracts; mild septicemia; states of weakened, suppressed or imbalanced immunity, including allergies and autoimmune disease; inflammatory and purulent conditions, including acne, abscess, furuncles training; envenomation. improve wound healing, increase resistance to infection and to increase connective tissue regeneration, inflamed skin conditions and bacterial infections		
<i>Equisetum arvense</i>	<i>Cola de caballo</i>	Cdc	Astringent, antidiarrhoeic, diuretic, healing effect, and emmenagogo agent	oral way	European
<i>Fabiana imbricata</i>	<i>Palo pichi</i>	Ppi	Diuretic	oral way	Argentine 3th edition
<i>Geoffroea decorticans</i>	<i>Chañar</i>	Cha	Hemorrhoids	oral way	Martínez G J et al., 2005 (16).
<i>Hamamelis virginiana</i>	<i>Hamamelis</i>	Ham	Astringent, antiinflammatory, haemostyptic; haemorrhoids, varicose veins; diarrhoea, mucous colitis; bruises and localised, inflamed swellings.	oral way/ topic use	Argentine 8th edition
<i>Hydrocotile asiática</i>	<i>Centella Asiática</i>	Cen	Assists cognitive function; facilitates venous irrigation and microcirculatory function;	oral way/ topic use	Argentine 8th edition

			modulates inflammation; moderates stress responses; calms the nervous system; regulates connective tissue function and promotes healing;		
<i>Lippia turbinata</i>	<i>Poleo</i>	Pol	Digestion, diuretic, tonic, emenagogo	oral way	Argentine annexed 2009
<i>Margyricarpus pinnatus</i>	<i>Yerba de la pediz</i>	Ype	Depurative blood, diuretic	oral way	
<i>Matricaria recutita</i>	<i>Manzanilla</i>	Man	Topical antiinflammatory effects, demulcent and astringent properties; Venotonic, antiedematous, haemorrhoids; Topically for haematoma, contusions, non-penetrating wounds and sports injuries involving oedema	oral way/ topic use	Argentine 8th edition
<i>Minthostachys mollis</i>	<i>Peperina</i>	Pep	Antibacterial, antihistaminic, hemostatic, antispasmodic and antidiarrheal	oral way/ topic use	Argentine 4th edition
<i>Peumus boldus</i>	<i>Boldo</i>	Bol	Soft laxative; antipyretic mechanism	oral way	Argentine 8th edition
<i>Plantago lanceolata</i>	<i>Plantago-llanten</i>	Pla	Gastritis, Ulcers, Diarrhea, Liver Diseases; Sores, Pustules (used locally)	oral way/ topic use	Spanish-European
<i>Ruta graveolens</i>	<i>Ruda</i>	Rud.	Anticonceptive	oral way	Argentine annexed 2009
<i>Smilax aspera</i>	<i>Zarzaparrilla</i>	Zar	Astringent, diuretic, cardiogenic and antisifilitic; healing;	oral way/ topic use	French- European
<i>Taraxacum officinale</i>	<i>Diente de león</i>	Ddl	Digestive and diuretic effect	oral way	British
<i>Thymus vulgaris</i>	<i>Tomillo</i>	Tom	Spasmolytic, antiseptic and	oral way/	Argentine 8th edition

			expectorant for respiratory conditions; antimicrobial and antiviral in topical application for the skin and mucous membranes	topic use	
<i>Urtica dioica</i>	<i>Ortiga</i>	Ort	Diarrhoea, dysentery, internal bleeding, chronic diseases of the colon, chronic skin eruptions, bladder irritations; topically for burns, wounds, nosebleeds, inflammation of the mouth or throat, joint pain (via the stinging of the skin around the joint); orally for skin rashes.	oral way/ topic use	European

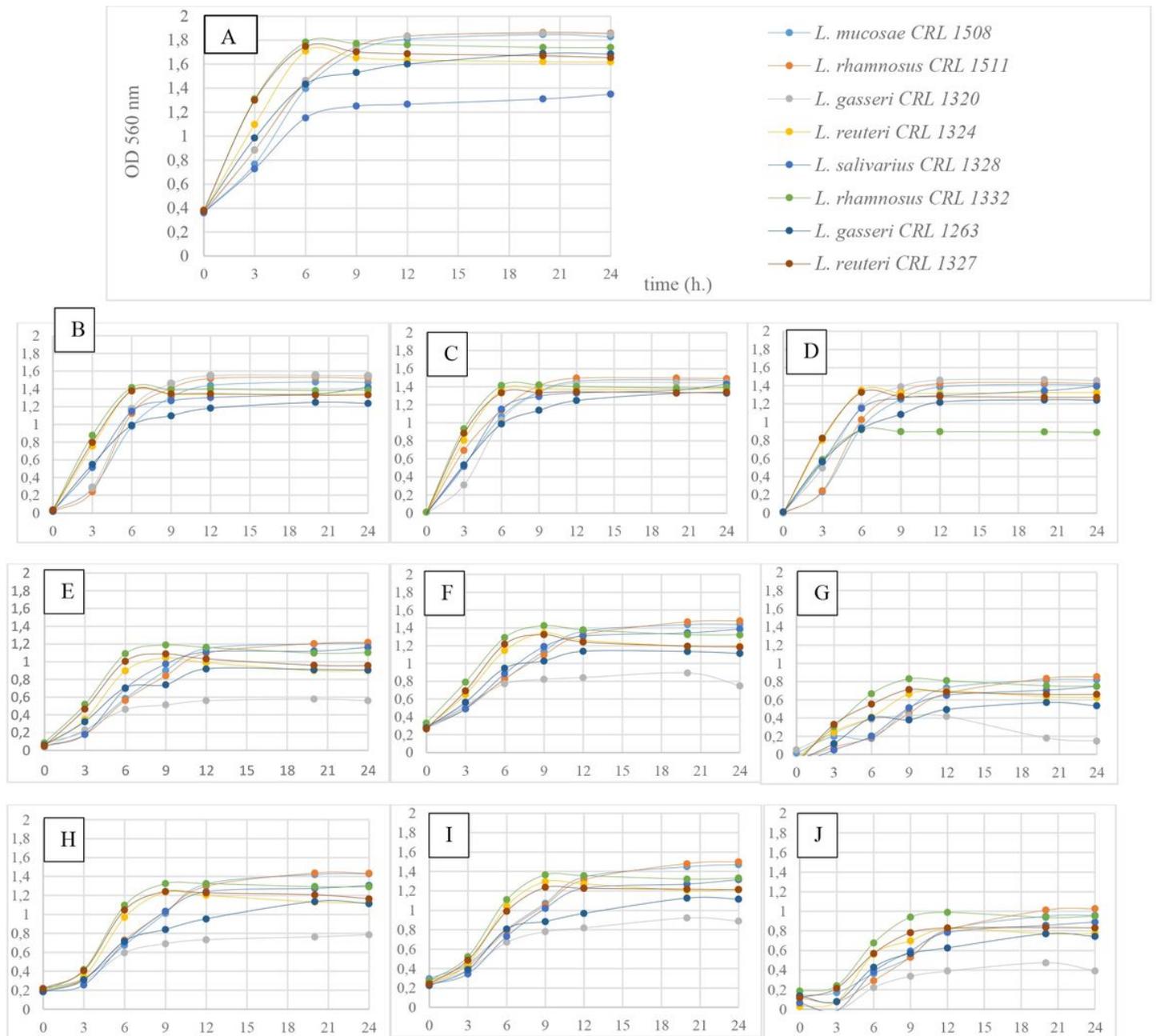
References: Application: different pharmacological effect of the natural compounds (4, 5, 14, 15, 16, 17). Uses: way of administration. Pharmacopoeia: year of publication and annexes in which they were accepted (15, 16, 17). All the extracts were obtained to FITOT laboratory, Tucuman- Argentina.

## Figures



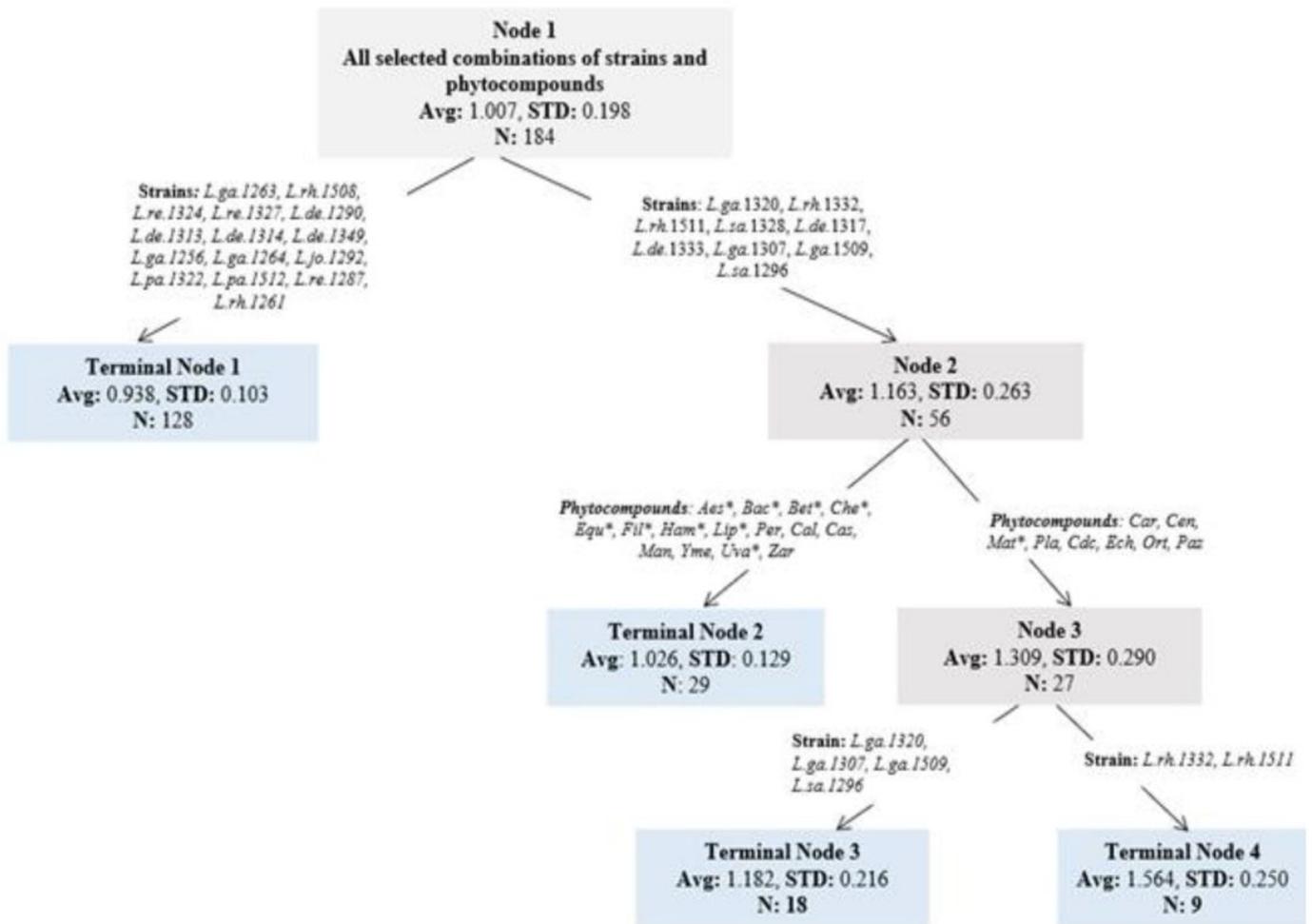
**Figure 2**

Kinetics of BVL growth added with different phytochemicals. Fig A-B: obligate homofermentative bacteria (OHe); Fig C- D: facultative heterofermentative (FHe), and Fig E-F: obligate homofermentative lactobacilli (OHo). The growth in standard media (MRS) of each strain of lactobacillus is represented by a dotted line. Different colors represent strains growth with added phytochemicals (Blue: “Betula”; Orange: “Lippia”; Board: “Ortiga”; Violet: “Carica”; Light Blue: “Centella”; Green: “Chelidonium”; Red: “Echinacea”).



**Figure 4**

Kinetic growth of BVL with different solid phytochemicals (solvent: ethanol 40%). A: control growth of BVL in standard MRS broth B: BVL growth in MRS+ 0.75 mg/ml *Allium sativa*. C: BVL growth in MRS+ 1.5 mg/ml *Allium sativa*. D: BVL growth in MRS+ 3 mg/ml *Allium sativa*. E: BVL growth in MRS+ 0.75 mg/ml *Hamamelis*. F: BVL growth in MRS+ 1.5 mg/ml *Hamamelis*. G: BVL growth in MRS+ 3 mg/ml *Hamamelis*. H: BVL growth in MRS+ 0.75 mg/ml *Uva ursi*. I: BVL growth in MRS+ 1.5 mg/ml *Uva ursi*. J: BVL growth in MRS+ 3 mg/ml *Uva ursi*



**Figure 5**

Cart tree of BVL and liquid phytocompounds obtained from the Statistical analysis carried out by SSPP, as indicated in materials and methods section. \*represents the extracts assayed as mixture.

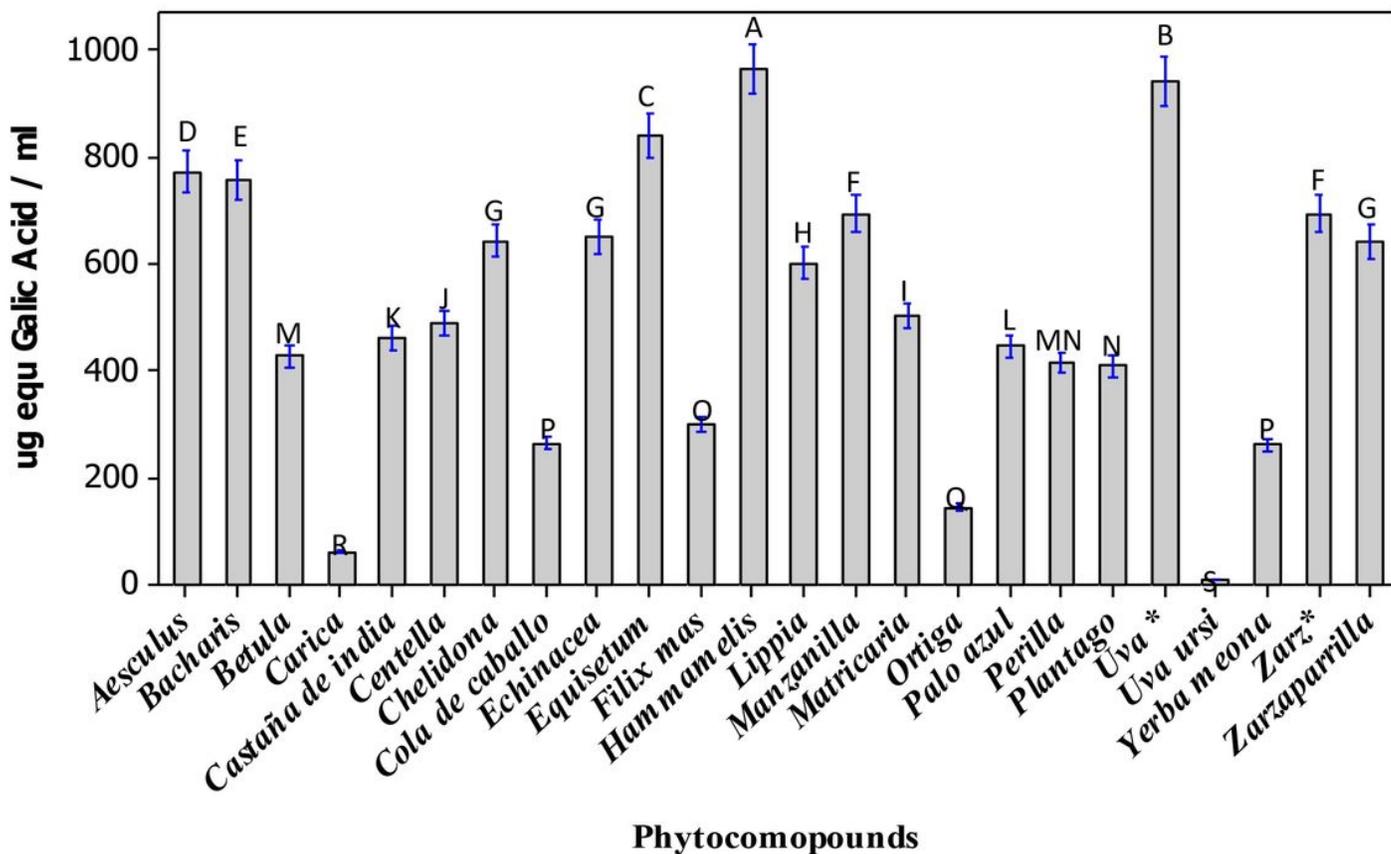


Figure 8

Quantification of phenolic compounds of phytoderivatives. Different letters represent significant differences ( $p < 0.005$ ).