frontiers in Sustainable Food Systems

ORIGINAL RESEARCH
published: 18 September 2018
doi: 10.3389/fsufs.2018.00058

Acid Lactic Bacteria as a Bio-Preservant for Grape Pomace Beverage

Juliana Furtado Dias[1], Beatriz Duarte Simbras[2], Carolina Beres[3], Karina Olbrich dos Santos[3], Lourdes Maria Correa Cabral[3] and Marco Antônio Lemos Miguel[2]*

[1] Department of Applied Nutrition, Federal University of the State of Rio de Janeiro, Rio de Janeiro, Brazil, [2] Laboratory of Food Microbiology, Institute of Microbiology Paulo de Góes, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil, [3] Brazilian Agricultural Research Corporation, Rio de Janeiro, Brazil

Probiotized juice represents an alternative to probiotic beverages derived from dairy products. Agricultural residue production represents an economical and environmental problem worldwide, its utilization to supplement a probiotic juice may be an applicable solution on food industry. Studies using fruit residues as an ingredient are not a novelty; however a definitive solution to this environmental problem has not yet been established. Therefore, the objective of this work was to propose a probiotized juice using lactic bacteria that, besides being a starter culture, can function as biopreservative and improve the stability of the final product. A fermented beverage was formulated using commercial grape juice, *Vitis vinifera Pinot noir* grape pomace and lactic bacteria. Pathogenic strains were used to simulate a potential protective effect on the beverage. Procedures were carried out according to the American Public Health Association and the results were expressed in media with standard deviation using statistical analyses performed by Prism. Lactic bacteria showed a cell growth of approximately 4 log CFU/ml. There was a significant decrease in pH values ($p < 0.05$) when pure grape juice was fermented. Grape juice supplemented with grape pomace from white winemaking was able to induce a higher growth of lactic bacteria population during fermentation, 5 log cycle CFU/mL, comparing to juice without supplementation. The beverage containing grape juice, water and pomace also presented growth on lactic bacteria population but *Lactobacillus rhamnosus* reached a higher concentration, of approximately 8 log cycle CFU/ml after 12 h fermentation. This was also observed when beverages were stored, only *L. rhamnosus* remained viable for 10 days at 10°C. All beverage samples co-inoculated with food borne pathogens, presented a stable and higher lactic bacteria population (2 log CFU/g) when compared to the added pathogen population. The final probiotic strain cells was above 8 log cycle CFU/mL, *L. rhamnosus* wasn't able to reduce significantly pathogenic population, however a bacteriostatic effect was observed. The probiotic beverage obtained represents a promising application for grape pomace. More studies are needed to investigate the causes and interactions of grape pomace compounds and lactic acid bacteria against food borne pathogens.

Keywords: lactic acid bacteria, probiotic, grape juice, grape pomace, Bio-Preservant, fermented beverage

OPEN ACCESS

Edited by:
Lorenzo Pastrana, Laboratório Ibérico Internacional de Nanotecnologia (INL), Portugal

Reviewed by:
Guadalupe Virginia Nevárez-Moorillón, Autonomous University of Chihuahua, Mexico
Miguel Cerqueira, Laboratório Ibérico Internacional de Nanotecnologia (INL), Portugal

****Correspondence:***
Juliana Furtado Dias
julifd@gmail.com

Specialty section:
This article was submitted to Sustainable Food Processing, a section of the journal Frontiers in Sustainable Food Systems

Received: *30 April 2018*
Accepted: *22 August 2018*
Published: *18 September 2018*

Citation:
Dias JF, Simbras BD, Beres C, dos Santos KO, Cabral LMC and Miguel MAL (2018) Acid Lactic Bacteria as a Bio-Preservant for Grape Pomace Beverage. Front. Sustain. Food Syst. 2:58. doi: 10.3389/fsufs.2018.00058

INTRODUCTION

Fruits are fundamental sources of water-soluble vitamins, phytosterols, dietary fibers, minerals and phytochemicals for human diet (Gebbers, 2007). Despite the increasing consumption, unfortunately daily intake of vegetables and fruits is estimated to be lower than the doses recommended by the World Health Organization (WHO), and Food and Agriculture Organization (FAO; Fan and Truelstrup Hansen, 2012). Grapes production is on top five fruits production worldwide, where the juice and wine industry drains 60% of this production (Muhlack et al., 2018).

The consumption of grapes and grape products has been related to health benefits such as: a reduction risk of cardiovascular disease and thrombosis (Ammollo et al., 2017), control of hypertension and dyslipidemia (Graf et al., 2010), besides antioxidant, anti-inflammatory, anti-aging and anti-diabetic properties (Castello et al., 2018); which are directly associated to polyphenol profile including flavonoids (anthocyanins, flavan-3-ols, flavonols, and flavanones) and non-flavonoids (phenolic acids, stilbenes and lignans; Soto et al., 2015) present mainly in the fruit peel and seed (Peixoto et al., 2018).

Consisting of skin and seeds, grape pomace is the most abundant by-product from the wine and grape juice industry (Syed et al., 2017). Approximately 10.8 million tons of grape pomace are produced in the world per year (Dwyer et al., 2014). The general composition of grape pomace was determinate by González-Centeno et al. (2010), which was: moisture varying from 50 to 72%, insoluble material with lignin content from 16.8 to 24.2%, and a content of <4% protein. However, this composition depends on grape variety and ripening state. In general, peptic substances are the main polymer-type constituent of grape pomace cell wall, ranging from 37 to 54% of cell wall polysaccharides, and cellulose is the second kind of cell wall polysaccharide varing from 27 to 37%. Those components can be classified as good quality dietary fiber. The fiber daily intake recommendation is of 25 g (Food Drug Administration (FDA) United States of America, 2013). In order to achieve this recommendation a fiber supplementation is needed. Most part of grape pomace is applied as a fertilizer or compost, however it is not appropriate, as the high levels of phenolic compounds present in grape pomace causes germination problems. The wine and grape juice by-products may be used for the production of high value extracts enriched in bioactive compounds, suitable for the functional and green market (Beres et al., 2016; Syed et al., 2017). However, the extract production still leaves a residue.

Nowadays there is an increase availability of grapes and their products necessary to attend a demanding market, that besides the nutritional aspect, search even more for the functional proprieties. Chemical composition and physical-chemical characteristics of grape and its by products, have been demonstrated to be a suitable substrate for microorganisms multiplication, such as lactic acid bacteria, acetic bacteria and yeasts, which are important for the production and deterioration on food products (Pastorkova et al., 2013; Campanella et al., 2017; Cho et al., 2018). This aspect opens an utilization possibility of winemaking industry by products on the formulation of fermented food.

The fermentation process results in the production of antimicrobial compounds and promote environmental modifications that inhibit pathogenic and deteriorating microorganisms on food matrix. Lactic acid bacteria (LAB) are one of the most used microorganisms, as they play a key role in food fermentations where they not only contribute to the development of the desired sensory properties in the final product but also to their microbiological safety. The antimicrobial effect of LAB is mainly related to the production of primary and secondary metabolites such as lactic and acetic acids, hydrogen peroxide, diacetyl, ethanol, antibiotics, phenolic compounds and nutrients competition (Cizeikiene et al., 2013; Bartkiene et al., 2017; Diepers et al., 2017). Some bacteria genders and species stand out as most used in industrial process, as *Lactobacillus casei*, *L. acidophilus,* and *Bifidobacterium* spp. Antimicrobial compounds and their concentration depends on the fermentation conditions and on the microorganism selected. However, these factors do not guarantee the production of a microbiological safe product. In some cases, fermented food need to be adjusted to attend sensorial consumers demands, which can represent the addition of new ingredients. This action, as well as a possible flaw during production may result in undesirable microorganisms survival on the final product. As a solution, starter cultures that present a more effective inhibitory aspect against pathogenic and deteriorating microorganisms can be used. This biopreservation using isolated bacteriocins or bacteriocins producing LAB represents an alternative for improving food safety and increase products shelf life. Bacteriocins, are a protein compound with antimicrobial activity produced by LAB, widely studied and applied as food biopreservative (Fan and Truelstrup Hansen, 2012). Studies have demonstrated good inhibition properties against pathogens indicators such as *L. monocytogenes* and *E. coli* on *in vitro* and *in vivo* approaches (Bartkiene et al., 2017; Diepers et al., 2017). Food biopreservation with natural and microbiological compounds may be a satisfactory approach solving economical losses due to microbial spoilage of raw materials and food products, to reduce the incidence of food borne illness (Cizeikiene et al., 2013). Besides the chemical preservatives reduction, an important issue that concerns the market is the offered variety of probiotic products. Probiotic products attend a consumer demand for healthy food as they may improve host intestinal microbiota and decrease pathogens amount that can develop disease (Azevedo et al., 2018). According to FAO/WHO (2006), probiotics are live microorganisms which when administered in adequate amounts induce health benefit on the host. These beneficial effects are mainly associated with the maintenance of a healthy gut microbiota, modulation of lactose intolerance, bowel function and gastrointestinal comfort, diarrhea, prevention and symptom alleviation, reduction of cholesterol levels and hypertension, regulation of immune response, amongst others (García-Ruiz et al., 2014). Usually probiotics are commercialized as dairy food, but the increasing number of consumers with restricted

consumption of dairy products has been changing this scenario (Mäkinen et al., 2016). The production and consumption of non-dairy probiotics has shown to be a growing and promising segment of this sector (Riviera-Espinoza and Gallardo-Navarro, 2010). Several processes apply the same microorganism as a starter culture and as a probiotic, which represents an advantage on food technology. Besides the use of natural preservatives, the production sustainability is relevant for the global environmental concern (Beres et al., 2017).

There is a need for a whole application of grape pomace in order to reduce considerably the by-product accumulation. In this case, this study aimed to produce a grape beverage fermented by probiotic LAB, supplemented with grape pomace from a white winemaking industry, in order to obtain a non-dairy probiotic product, supplemented with dietary fiber from grape pomace.

MATERIALS AND METHODS

Beverage Components

The fermented beverage was formulated using commercial organic grape juice (Casa de Bento®), and 5% (m/v) of *Vitis vinifera Pinot noir* grape pomace from white wine making process (2011 harvest) stored at −18°C. No mechanical procedure was performed on grape pomace. No chemical additives were added. Pasteurization (85°C/15 min) was performed in water bath with agitation (60 rpm) for all formulations.

Bacterial Strains

Strains were obtained from Food Microbiology Laboratory (Institute of Microbiology Paulo de Góes) culture collection. Lactic acid bacteria (LAB) used in this study were *Lactobacillus casei* subsp. *casei* ATCC 393 (*L. casei*); *Lactobacillus casei* subsp. *rhamnosus* ATCC 7469; *Lactobacillus delbrueckii* subsp. *delbrueckii* ATCC 9649. The pathogens strains were *Salmonella enterica* subsp. *enterica serovar* Enteritidis ATCC 13076; *Listeria monocytogenes* and *Escherichia coli* ATCC 25922. LAB strains were stored in Brain and Heart infusion (BHI) supplemented with 2% yeast extract and cultivated in de Man, Rogosa & Sharp (MRS, Merck®), for 24–48 h, at 37°C in microaerophylic condictions. *E. coli* was incubated in BHI for 18–24 h at 37°C.

Beverage Formulation and Microbial Inoculum

Three experiments were conducted sequentially aiming to determine the lactobacilli strain most able to growth in pure grape juice (Experiment 1), to determine the ability of the selected strain to growth in pure juice added by grape pomace (Experiment 2), and to verify the fermentation behavior of the selected strain in a lower cost beverage formulated with diluted grape juice, sugar and grape pomace (Experiment 3). LAB and pathogens cultures were activated in MRS and BHI agar, respectively. Three different beverages were fermented using LAB: pure juice; pure juice plus grape pomace; and diluted juice plus grape pomace. The fermentation was conducted separately and equally in 200 mL sterile flasks, with 100 mL of each formulation, at 37°C for 24 h. The inoculum for each fermentation is indicated on **Table 1**. During fermentation, 1 mL was aliquoted for bacteria quantification each 4 or 6 h. The pH was monitored every 6 h with pHmeter (Medical MPA-210).

TABLE 1 | Lactic acid bacteria and pathogens inoculums concentration used on different beverage formulations.

Beverage formulations	Culture inoculum (CFU/mL)					
	LR	LC	LD	SE	LM	EC
Pure grape juice (Experiment 1)	10^4	–	–	–	–	–
Pure grape juice (Experiment 1)	–	10^4	–	–	–	–
Pure grape juice (Experiment 1)	–	–	10^4	–	–	–
Grape juice and grape pomace (Experiment 2)	10^6	–	–	–	–	–
Diluted grape juice, sugar and grape pomace (Experiment 3)	10^6	–	–	–	–	–
Diluted grape juice, sugar and grape pomace (Experiment 4)	10^6	–	–	10^6	–	–
Diluted grape juice, sugar and grape pomace (Experiment 4)	10^6	–	–	–	10^6	
Diluted grape juice, sugar and grape pomace (Experiment 4)	10^6	–	–	–	–	10^6

LR, Lactobacillus casei subsp. rhamnosus ATCC 7469; LC, Lactobacillus casei subsp. casei ATCC 393 (L. casei); LD, Lactobacillus delbrueckii subsp. delbrueckii ATCC 9649; SE, Salmonella enterica subsp. enterica serovar Enteritidis ATCC 13076; LM, Listeria monocytogenes; EC, Escherichia coli ATCC 25922; CFU, colony-forming unit; mL, mililiter.

Pure juice (100 mL) was fermented using 10^4 CFU/mL of *L. casei* (LC), *L. rhamnosus* (LR) and *L. delbrueckii* (LD), in order to obtain the most suitable strain (Experiment 1). The culture which presented the highest growth on juice was used (10^6 CFU/mL) to ferment the beverage formulated with grape juice and grape pomace (5% m/v; Experiment 2). In an attempt to produce a lower cost beverage a diluted juice was prepared with 50% water, 10% sugar and it was added 5% (m/v) grape pomace. To determine the culture ability to ferment the more economical beverage two cultures, *L. casei* and *L. rhamnosus* were tested, (10^6 CFU/mL; Experiment 3).

The potential biopreservative effect was tested in Experiment 4. This test was performed in diluted grape juice prepared with 50% water, 10% sugar and it was added 5% (m/v) of grape pomace. The LAB culture that had the best development in Experiment 1 was used (10^6 CFU/mL) to compete against three pathogens used to simulate a bacterial contamination, which were *E. coli*, *S. Enteritidis* and *L. monocytogenes*. Pathogenic inoculums (**Table 1**) were added in the fermented grape juice (after fermentation). In a non-fermented grape juice the pathogenic culture was inoculated simultaneously with the starter culture (before fermentation), in order to compare the LAB protective effect in different contamination conditions. Positives control were pure grape juice inoculated with the LAB culture and the three pathogens individually. Negative control was grape juice with no microorganisms.

Microbiological Characterization

The strains were quantified using serial dilution and spread plate cultivation. *L. monocytogenes* was cultivated on Agar

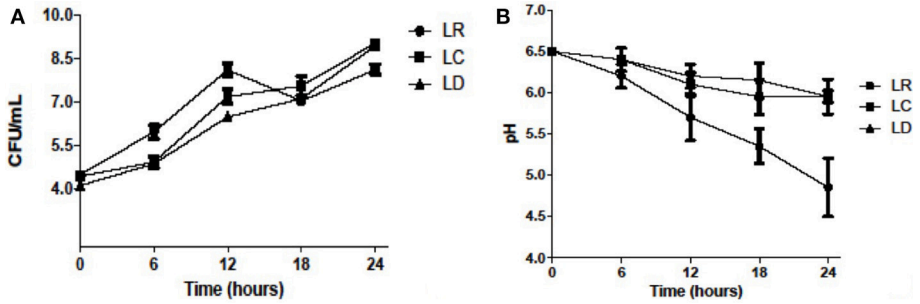

FIGURE 1 | Cell viability of *Lactobacillus casei* (LC), *Lactobacillus delbrueckii* (LD), *Lactobacillus rhamnosus* (LR) **(A)** and pH values **(B)** during pure grape juice fermentation for 24 h at 37°C.

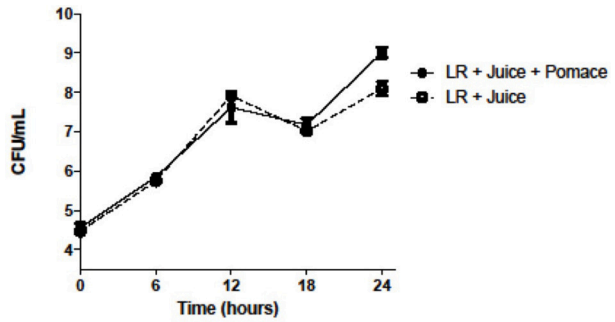

FIGURE 2 | Comparison of *Lactobacillus rhamnosus* (LR) viability during 24 h fermentation of grape juice with and without grape pomace at 37°C.

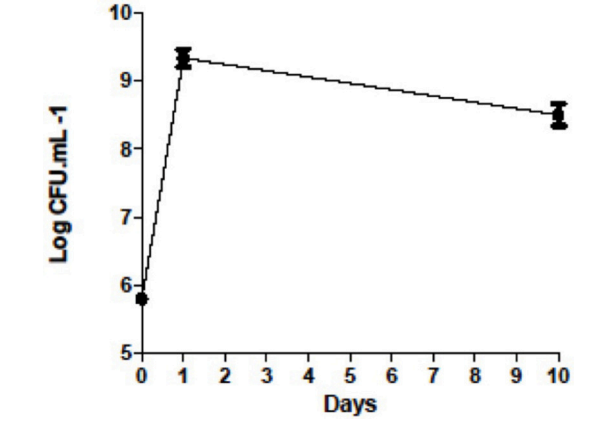

FIGURE 3 | *Lactobacillus rhamnosus* viability during diluted grape beverage 24 h fermentation. Initial inoculum was 10^6 CFU/mL Formulation was storage for10 days at 4°C.

Listeria according to Ottaviani and Agosti medium, *E. coli* detection was performed in Eosin Methylene Blue (EMB) and *S. Enteritidis* was incubated on *Salmonella-Shigella* Agar (SS Agar). Microaerophilic atmosphere and MRS Agar was used for lactobacilli strains incubation. Procedures were carried out according to the American Public Health Association (APHA, 2001), and analyses were performed in triplicate. The pH of each formulation was determined with pHmeter (Medical MPA-210), every 6 h.

Beverage Stability

The probiotic culture stability was tested on the diluted beverage, during 10 days of storage at 4°C. A stable probiotic counting indicates a possible food application as there is a need to assure a minimum cell number during shelf life. Lactic acid bacteria quantification were analyzed after 12 and 24 h of inoculums of pathogenic microrganisms. Than at 4, 7, 14, 21 and 28 days after inoculums (Experiment 4).

Statistical Analysis

The results were expressed in media with standard deviation in order to verify the difference between samples, an analysis of variance (ANOVA) was applied followed by Tukey test or *t*-test at a significant level of 5% ($p < 0.05$) All statistical analyses were performed using Prism 6.0 software (GraphPad Software, Inc. 2006).

RESULTS

Even though the statistical analysis has not shown any significant difference ($p < 0.05$) in the fermentation capacity of the three analyzed cultures, *Lactobacillus rhamnosus* manage to achieve a higher cell number and more rapidly reduce pH, when compared to LC and LD (**Figure 1**). This culture was also the only able to reduce pH value below 5.5, which is normally used in fermented beverage production. Due to that, only LR were tested in the complementary trials. The LR multiplication in juice with an without pomace resulted in a very similar growing curve, where final cell count was between 10^8 and 10^9 CFU/mL, which represents a total increase of 4 log cycles. However, even if no statistical difference was observed, a final cell count in juice with pomace was 1 log cycle higher than in juice without pomace. When LR were cultivated in a formulation with pomace, differing only in juice and water ratio, a higher growth were observed in the more diluted formulation, which achieved a final count cell proper to probiotic products (10^9 CFU/mL; **Figure 2**). It was also observed a fast exponential growth, product acidification and stability during 10 days of storage, maintaining cell count

TABLE 2 | Cell viability of pathogens added after and before fermentation in intentionally contaminated grape beverages fermented by *Lactobacillus rhamnosus* and stored for 28 at ±4°C(first latter indicates cell count difference a long time using Anova; second latter indicates cell count difference comparing to control using *t*-test.

Sample	Culture	Viability							
		Time (day)	Time (day)	Time (day)	Time (day)	Time (day)	Time (day)	Time (day)	Time (day)
		0	0.5	1	4	7	14	21	28
Juice with isolated culture	LR	$8.52 \pm 0.07^{a,a}$	$8.28 \pm 0.06^{a,a}$	$8.50 \pm 0.05^{a,a}$	$8.52 \pm 0.09^{a,a}$	$8.43 \pm 0.05^{a,a}$	$8.56 \pm 0.07^{a,a}$	$8.56 \pm 0.02^{a,a}$	$8.35 \pm 0.05^{a,a}$
	EC	$6.39 \pm 0.07^{a,a}$	$8.13 \pm 0.04^{b,a}$	$8.22 \pm 0.10^{b,a}$	$8.37 \pm 0.10^{b,a}$	$8.59 \pm 0.01^{b,a}$	$8.65 \pm 0.06^{b,a}$	$8.17 \pm 0.04^{a,a}$	$8.32 \pm 0.29^{a,a}$
	SE	$7.35 \pm 0.10^{a,a}$	$8.11 \pm 0.00^{b,a}$	$8.11 \pm 0.00^{b,a}$	$7.83 \pm 0.02^{b,a}$	$8.01 \pm 0.03^{b,a}$	$7.52 \pm 0.04^{a,a}$	$7.65 \pm 0.06^{a,a}$	$7.65 \pm 0.06^{a,a}$
	LM	$7.23 \pm 0.06^{a,a}$	$7.28 \pm 0.02^{a,a}$	$7.27 \pm 0.02^{a,a}$	$6.89 \pm 0.00^{a,a}$	$6.95 \pm 0.42^{a,a}$	$6.86 \pm 0.12^{a,a}$	$6.80 \pm 0.21^{a,a}$	$6.13 \pm 0.39^{a,a}$
BEFORE FERMENTATION									
Juice with LR and EC	LR	$7.35 \pm 0.07^{a,a}$	$8.24 \pm 0.02^{a,a}$	$8.30 \pm 0.01^{a,a}$	$8.36 \pm 0.08^{a,a}$	$8.28 \pm 0.08^{a,a}$	$8.30 \pm 0.04^{a,a}$	$8.20 \pm 0.02^{a,a}$	$8.41 \pm 0.01^{a,a}$
	EC	$7.08 \pm 0.14^{a,a}$	$7.95 \pm 0.04^{a,a}$	$7.96 \pm 0.05^{a,a}$	$8.34 \pm 0.09^{a,a}$	$6.46 \pm 0.01^{a,a}$	$8.28 \pm 0.01^{a,a}$	$7.68 \pm 0.04^{a,a}$	$7.03 \pm 0.05^{a,a}$
Juice with LR and SE	LR	$6.98 \pm 0.02^{a,a}$	$8.49 \pm 0.06^{a,a}$	$8.45 \pm 0.04^{a,a}$	$5.23 \pm 0.33^{a,a}$	$6.03 \pm 0.01^{a,a}$	$8.23 \pm 0.08^{a,a}$	$8.18 \pm 0.05^{a,a}$	$8.33 \pm 0.03^{a,a}$
	SE	$7.45 \pm 0.03^{a,a}$	$7.85 \pm 0.08^{a,a}$	$7.81 \pm 0.05^{a,a}$	$7.56 \pm 0.11^{a,a}$	$7.38 \pm 0.12^{a,a}$	$6.22 \pm 0.10^{a,a}$	$6.95 \pm 0.00^{a,a}$	$6.66 \pm 0.04^{a,a}$
Juice with LR and LM	LR	$7.09 \pm 0.02^{a,a}$	$8.57 \pm 0.04^{a,a}$	$8.47 \pm 0.01^{a,a}$	$8.36 \pm 0.07^{a,a}$	$8.58 \pm 0.01^{a,a}$	$8.58 \pm 0.04^{a,a}$	$8.72 \pm 0.04^{a,a}$	$8.18 \pm 0.04^{a,a}$
	LM	$5.49 \pm 0.04^{a,a}$	$7.27 \pm 0.03^{a,a}$	$7.28 \pm 0.02^{a,a}$	$6.89 \pm 0.00^{a,a}$	$7.10 \pm 0.11^{a,a}$	$6.93 \pm 0.04^{a,a}$	$6.80 \pm 0.13^{a,a}$	$6.93 \pm 0.02^{a,a}$
AFTER FERMENTATION									
Juice with LR and EC	LR	$7.30 \pm 0.03^{a,a}$	$8.45 \pm 0.05^{a,a}$	$8.58 \pm 0.03^{a,a}$	$8.44 \pm 0.06^{a,a}$	$8.27 \pm 0.04^{a,a}$	$8.36 \pm 0.09^{a,a}$	$8.36 \pm 0.00^{a,a}$	$8.35 \pm 0.04^{a,a}$
	EC	–	$6.88 \pm 0.06^{a,b}$	$6.88 \pm 0.06^{a,b}$	$6.61 \pm 0.02^{a,b}$	$6.46 \pm 0.01^{a,b}$	$6.91 \pm 0.00^{a,b}$	$6.65 \pm 0.06^{a,b}$	$5.05 \pm 0.02^{a,b}$
Juice with LR and SE	LR	$7.16 \pm 0.14^{a,a}$	$6.15 \pm 0.21^{a,a}$	$7.39 \pm 0.04^{a,a}$	$8.31 \pm 0.04^{a,a}$	$4.99 \pm 0.07^{a,a}$	$8.55 \pm 0.06^{a,a}$	$8.35 \pm 0.08^{a,a}$	$8.70 \pm 0.04^{a,a}$
	SE	–	$5.15 \pm 0.10^{a,b}$	$5.15 \pm 0.10^{a,b}$	$5.07 \pm 0.10^{a,b}$	$4.79 \pm 0.04^{a,b}$	$7.61 \pm 0.00^{a,b}$	$7.06 \pm 0.01^{a,b}$	$7.24 \pm 0.04^{a,b}$
Juice with LR and LM	LR	$7.47 \pm 0.00^{a,a}$	$8.33 \pm 0.03^{a,a}$	$8.49 \pm 0.03^{a,a}$	$8.32 \pm 0.03^{a,a}$	$8.32 \pm 0.03^{a,a}$	$8.47 \pm 0.00^{a,a}$	$8.54 \pm 0.02^{a,a}$	$8.33 \pm 0.04^{a,a}$
	LM	–	$7.56 \pm 0.05^{a,b}$	$7.56 \pm 0.15^{a,b}$	$7.64 \pm 0.07^{a,b}$	$7.62 \pm 0.02^{a,b}$	$7.12 \pm 0.06^{a,b}$	$6.90 \pm 0.08^{a,b}$	$6.58 \pm 0.02^{a,b}$

Different values ($p < 0.5$) are shown using different letters)

around 10^8-10^9 CFU/mL (**Figure 3**). LR culture inoculated in grape juice had no significant difference in cell count during storage conditions, as presented in **Table 2**. A decrease from 5.5 to 4.6 on pH values was observed, after 28 days storage. Pathogens and LR were intentionally inoculated at the same time on beverage to evaluate a potential inhibitory aspect, the population behavior in the juice along the period was slightly different. *E. coli* had an increase of 2 log cycles from inoculum day (6.39 ± 0.07 CFU/mL) to final day (8.32 ± 0.29 CFU/mL). On the contrary *L. monocytogenes* had an initial inoculum of 7.23 ± 0.10 CFU/mL and after 28 days the population decreased 1 log cycle (6.13 ± 0.39 CFU/mL). *S. enteretidis* did not show any difference from the initial inoculum, in comparison to the last day of storage. All three samples inoculated with pathogens presented a decrease in the pH value. The pathogens were also inoculated after grape juice 12 h fermentation at 37°C using *L. rhamnosus* as a starter culture. *E. coli* and *L. monocytogenes* presented a decrease of approximately 1 log cycle each, however *S. enteretidis* increased 2 log cycles. The pH values decreased from 5.5 to 4.8–3.9 in all three samples (**Table 2**). The same *L. rhamnosus* population behavior was observed before and after fermentation, cell count was stable even with the pathogen presence. This result suggests a bio-preservative, and a bacteriostatic effect from *L. rhamnosus* against foodborne pathogens, mainly against *L. monocytogenes*.

DISCUSSION

The consumer demand for non-dairy beverages with high functional value is increasing together with the trend of vegetarianism and the prevalence of lactose intolerance and allergies Coda et al., 2012. This work proposed a fermented beverage with probiotic LAB and supplemented with grape pomace. All probiotic bacteria tested were capable to ferment the integral grape juice used with no statistical difference.

All LAB were able to reduce pH, however LR had the higher pH reduction which was to <5.0. Organic acids, hydrogen peroxide and bacteriocins produced by LAB during fermentation can decreasethe pH in solution (Albano et al., 2007). The ability to decrease pH is related to the bio-preservative proprieties of LAB to inhibit spoilage microorganisms growth.

During processing or storage of the probiotic product the viability and functionality should not be affected (Diepers et al., 2017). In this way the probiotic beverage formulated with juice,

water, sugar and pomace was storage for 10 days at 10°C, LR was considered suitable for the study purpose as it remained stable with a count cell of 9 CFU/mL during the storage period, A functional beverage made with a mixture of rice and barley and concentrated red grape must were fermented with selected strains of *L. plantarum*, which remained viable at 8.5 log UFC g-1 throughout storage at 4°C for 30 days Coda et al., 2012. Probiotics not only have to survive at high cell numbers but do not have to impact unsuitable modifications of the sensory properties of the fruit juice (Mousavi et al., 2010).

After 24 h fermentation using LR on pure commercial juice with and without grape pomace cell count on juice supplemented with grape pomace was higher than in the juice without supplementation. Grape pomace is rich in polysaccharides that are classified as dietary fiber and can act as a prebiotic. The addition of prebiotic fiber led to a significant increase of antioxidant capacity, may contribute to health beneficial properties against several life style disorders such as diabetes, cancer, ulcer and atherosclerosis (Cassani et al., 2016; Kowalska et al., 2017). The use of fruit pomace in functional food preparation may lead to an improvement for the probiotic cell stability and growth. Another technological improvement of by-product utilization was demonstrated by (Di Cagno et al., 2011), where white grape juice and *Aloe vera* extract were mixed with red or green fruits and vegetables and were subjected to fermentation with mixed starters cultures, consisting of *L. plantarum*, *W. cibaria* and *L. pentosus* strains. Lactic acid fermentation by selected starters positively affected the content of antioxidant compounds and enhanced the sensory attributes.

The tested pathogenic were detected as food contaminants in different sources such as; orange juice (Patil et al., 2009), cabbage (Fukuyama et al., 2009), lettuce and parsley (Sengun, 2013). Öncül and Karabiyikli (2016) determined that *E. coli* and *Salmonella spp*. were part of initial microbiota of grape products. In this case, a natural preservative against these pathogens became interesting for a product with grape pomace and juice. The bio-preservative effect of LR was tested after co-inoculation a bacteriostatic action was observed. When the co-inoculation was performed before fermentation, *E. coli* was stable and *S. enteridis* decrease. *L. monocytogenes* presented an increased on cell count after 28 days when co-inoculated with LR. *Listeria monocytogenes* is the causative agent of listeriosis, a severe disease in humans and one of the most significant foodborne diseases in industrialized countries, is a psychrotrophic microorganism widely distributed in the environment that can grow at refrigerated temperatures and is highly acidic and salt-tolerant. Coelho et al. (2014) inoculated cheese with *L. lactis* and *L. monocytogenes*. After 7 days of refrigeration, a reduction of two log units was observed. *E. faecalis* strains decreased *L. monocytogenes* counts by 3 or 4 log units compared to the control. It is possible to determine that the co-inoculation of LAB and the pathogen can inhibit the cell count growth. However, inhibiting mechanism needs to be further examined. When LR was co-inoculated after fermentation both *E. coli* and *L. monocytogenes* had a decrease in cell count. After 28 days storage, all samples presented a pH decrease, in this case is possible to affirm that the lower pH was not able to decrease the pathogen population. In another study, Cizeikiene et al. (2013) tested *Lactobacillus sakei* KTU05-6, *Pediococcus acidilactici* KTU05-7, *Pediococcus pentosaceus* KTU05-8, KTU05-9, and KTU05-10 against *Listeria monocytogenes*, *E. coli* ATCC 25922, and *Salmonella typhimurium*. And *E. coli* ATCC 25922 was only inhibited by *P. Acidilactici*. Besides LAB metabolites effects, natural organic acids from grape pomace could influence in foodborne pathogens inhibition. Grapes present large amounts of phenolic compounds, mainly flavonoids, which are able to damage cytoplasmic membrane promoting a deregulation of electron flow, leading to cell death (Öncül and Karabiyikli, 2016). A better result was observed when pathogens were inoculated after LR fermentation on grape juice, this may be due to the induction on structural degradation on plant cell walls resulting in the liberation of phenolic compounds (Cho et al., 2018). The deleterious effect on microbial cell membrane was observed on *Clostridium histolyticum* by Cueva et al. (2013), who also demonstrated that this negative effect does not occur against LAB, on the contrary, the study showed that there was a stimulated growth of *Lactobacillus* and *Entercoccus* when grape pomace was used.

CONCLUSION

This study suggested a food application for an agricultural by-product using a probiotic bacteria as a bio-preservative. LABs used could grow in the grape juice with and without pomace supplementation. The probiotic grape beverage containig juice supplemented with grape pomace presented storage stability and a bacteriostatic action against pathogens indicators. The use of LAB fermentation to produce a non-dairy probiotic product may solve two market problems: reduction of environmental impact and market diversification for probiotic foods. Most studies use grape pomace extracts, what does not reduce significantly the residue amount and non-fermented juices, what is more expensive and may not provide biopreservation characteristics. In this case the whole pomace was used as source of polysaccharides that represent a dietary fiber supplementation, with a possible prebiotic function increasing the probiotic population.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

ACKNOWLEDGMENTS

The authors would like to thank the Brazilian agencies *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES) and *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq) for their financial support. The authors also thank Aurora Winery Ltda for supplying the grape pomace.

REFERENCES

Albano, H. M., Oliveira, R., Arso, N., Cubero, T., and Hogg, P. (2007). Antilisterial activity of lactic acid bactéria isolated from "Alheira" (traditional Portuguese fermented sausage). *In situ* assays. *Meat Sci*. 76, 796–800. doi: 10.3168/jds.2009-3000

Ammollo, C. T., Semeraro, F., Milella, R. A., Antonacci, D., Semeraro, N., and Colucci, M. (2017). Grape intake reduces thrombin generation and enhances plasma fibrinolysis. Potential role of circulating procoagulant microparticles. *J. Nutri. Biochem*. 50, 66–73. doi: 10.1016/j.jnutbio.2017.08.012

APHA (2001). *Compendium of Methods for the Microbiological Examination of Foods*. *4th Edn*. Washington, DC: APHA.

Azevedo, P. O. S., Aliakbarian, B., Casazza, A. A., LeBlanc, J. G., and Perego, P., Oliveira, R. P. S. (2018). Production of fermented skim milk supplemented with different grape pomace extracts: effect on viability and acidification performance of probiotic cultures. *Pharmanutrition* 6, 64–68. doi: 10.1016/j.phanu.2018.03.001

Bartkiene, E., Bartkevics, V., Mozuriene, E., Krungleviciute, V., Novoslavskij, A., Santini, A., et al. (2017). The impact of lactic acid bacteria with antimicrobial properties on biodegradation of polycyclic aromatic hydrocarbons and biogenic amines in cold smoked pork sausages. *Food Control* 71, 285–292. doi: 10.1016/j.foodcont.2016.07.010

Beres, C., Costa, G. N. S., Cabezudo, I., Silva-James, N. K., Teles, A. S. C., Cruz, A. P. G., et al. (2017). Toward integral utilization of grape pomace from winemaking process: a review. *Waste Manag*. 68, 581–594. doi: 10.1016/j.wasman.2017.07.017

Beres, C., Simas-Tosin, F. F., Cabezudo, I., Freitas, S. P., Iacomini, M., Mellinger-Silva, C., et al. (2016). Antioxidant dietary fibre recovery from Brazilian Pinot noir grape pomace. *Food Chem*. 201, 145–152. doi: 10.1016/j.foodchem.2016.01.039

Campanella, D., Rizzello, C. G., Fasciano, C., Gambacorta, G.,Pinto, D., Marzani, B., et al. (2017). Explotation of grape marc as a functional substrate for lactic acid bacteria and bifidobacteria growth and enhanced antioxidant activity. *Food Microbiol*. 65, 25–35. doi: 10.1016/j.fm.2017.01.019

Cassani, L., Tomadoni, B., Viacava, G., Ponce, A., and Moreira, M. R. (2016). Enhancing quality attributes of fiber-enriched strwberry juice by application of vanillin or geraniol. *LWT Food Sci. Technol*. 72, 90–98. doi: 10.1016/j.lwt.2016.04.037

Castello, F., Costabile, G., Bresciani, L., Tassotti, M., Naviglio, D., Luongo, D., et al. (2018). Bioavailability and pharmacokinetic profile of grape pomace phenolic compounds in humans. *Arch. Biochem. Biophys*. 646, 1–9. doi: 10.1016/j.abb.2018.03.021

Cho, Y., Kim, D., Jeong, D., Seo, K., Jeong, H., Lee, H., et al. (2018). Characterization of yeasts isolated from kefir as a probiotic and its synergic interaction with the wine byproducts grape seed flour/extract. *LWT Food Sci. Technol*. 90, 535–539. doi: 10.1016/j.lwt.2018.01.010

Cizeikiene, D., Juodeikiene, G., Paskevicius, A., and Bartkiene, E. (2013). Antimicrobial activity of lactic acid bacteria against pathogenic and spoilage microorganism isolated from food and their control in wheat bread. *Food Control* 31, 539–545. doi: 10.1016/j.foodcont.2012.12.004

Coda, R., Lanera, A., Trani, A., Gobbetti, M., and Di Cagno, R. (2012). Yogurt-like beverages made of a mixture of cereals, soy and grape must: microbiology, texture, nutritional and sensory properties. *Int. J. Food Microbiol*. 155, 120–127. doi: 10.1016/j.ijfoodmicro.2012.01.016

Coelho, M. C., Silva, C. C. G., Ribeiro, S. C., Dapkevicius, M. L. N. E., and Rosa, H. J. D. (2014). Control of Listeria monocytogenes in fresh cheese using protective lactic acid bacteria. *Int. J. Food Microbiol*. 191, 53–59. doi: 10.1016/j.ijfoodmicro.2014.08.029

Cueva, C., Sanchez-Patan, F., Monagas, M., Walton, G. E., Gibson, G. R., and Martin-Alvarez, P. J. (2013). *In vitro* fermentation of grape seed flavan-3-ol fractions by human faecal microbiota: changes in microbial groups and phenolic metabolites. *FEMS Microbiol. Ecol*. 83, 792–805. doi: 10.1111/1574-6941.12037

Di Cagno, R., Minervini, G., Rizzello, C. G., De Angelis, M., and Gobbetti, M. (2011). Effect of lactic acid fermentation on antioxidant, texture, color and sensory properties of red and green smoothies. *Food Microbiol*. 28, 1062–1071. doi: 10.1016/j.fm.2011.02.011

Diepers, A., Krömker, V., Zinke, C., Wente, N., Pan, L., Paulsen, K., et al. (2017). *In vitro* ability of lactic acid bacteria to inhibit mastitis-causing pathogens. *Sust. Chem. Pharm*. 5, 84–92. doi: 10.1016/j.scp.2016.06.002

Dwyer, K., Hosseinian, F., and Rod, M. (2014). The market potential of grape waste alternatives. *J. Food Res*. 3, 91–106. doi: 10.5539/jfr.v3n2p91

Fan, L., and Truelstrup Hansen, L. (2012). "Fermentation and biopreservation of plantbased foods with lactic acid bacteria," in *Handbook of Plant Based Fermented Food and Beverage Technology, 2nd Edn.* ed Y. H. Hui (Boca Raton, FL: CRC Press), 35–48.

FAO/WHO (2006). *Probiotics in Food: Health and Nutritional Properties and Guidelines for Evaluation*. FAO Food Nutrition Pap. 85. Rome: World Health Organization and Food and Agriculture Organization of the United Nations.

Food and Drug Administration (FDA) United States of America (2013). *Guidance for Industry: A Food Labeling Guide (14. Appendix F: Calculate the Percent Daily Value for the Appropriate Nutrients)*. U.S. Department of Health and Human Services.

Fukuyama, S., Watanabe, Y., Kondo, N., Nishinomiya, T., Kawamoto, S., and Isshiki, K. (2009). Efficiency of sodium hypochlorite and calcinated calcium in killing *Escherichia coli* O157:H7, *Salmonella spp*., and *Staphylococcus aureus* attached to freshly shredded cabbage. *Biosci. Biotechnol. Biochem*. 73, 9–14. doi: 10.1271/bbb.70722

García-Ruiz, A., Llano, D. G., Esteban-Fernández, A., Requena, T., Bartolomé, B., and Moreno-Arribas, M. V. (2014). Assessment of probiotic properties in lactic acid bactéria isolated from wine. *Food Microbiol*. 44, 220–225. doi: 10.1016/j.fm.2014.06.015

Gebbers, J. O. (2007). Atherosclerosis, cholesterol, nutrition, and statins a critical review. *German Med. Sci*. 5, 1–11.

González-Centeno, M. R., Rosselló, C., Simal, S., garau, M. C., and López, F., Femenia, A. (2010). Physico-chemical properties of cell wall materials obtained from ten grape varieties and their byproducts: grape pomace and stems. *LWT Food Sci. Technol*. 43, 1580–1586. doi: 10.1016/j.lwt.2010.06.024

Graf, B. L., Raskin, I., Cefalu, W. T., and Ribnicky, D. M. (2010). Plant-derived therapeutics for the treatment of metabolic syndrome. *Curr. Opin. Invest. Drugs* 11, 1107–1115.

Kowalska, H., Czajkowska, K., Cichowska, J., and Lenart, A. (2017). What's new in biopotential of fruit and vegetable by-products applied in the food processing industry. *Trends Food Sci. Technol*. 67, 150–159. doi: 10.1016/j.tifs.2017.06.016

Mäkinen, O. E., Wanhalinna, V., Zannini, E., and Arendt, E. K. (2016). Foods for special dietary needs: non-dairy plant-based milk substitutes and fermented dairy-type products. *Crit. Rev. Food Sci. Nutri*. 56, 339–349. doi: 10.1080/10408398.2012.761950

Mousavi, Z. E., Mousavi, S. M., Razavi, S. H., Emam-Djomeh, Z., and Kiani, H. (2010). Fermentation of pomegranate juice by probiotic lactic acid bacteria. *World J. Microbiol. Biotechnol*. 27, 123–128. doi: 10.1007/s11274-010-0436-1

Muhlack, R. A., Potumarthi, R., and Jeffery, D. W. (2018). Sustainable wineries throught waste valorisation: a review of grape marc utilisation for value-added products. *Waste Manag*. 72, 99–118. doi: 10.1016/j.wasman.2017.11.011

Öncül, N., and Karabiyikli, Ş. (2016). Survival of foodborne pathogens in unripe grape products. *LWT Food Sci. Technol*. 74, 168–175. doi: 10.1016/j.lwt.2016.07.043

Pastorkova, E., Zakova, T., Landa, P., Novakova, J., Vadhejch, J., and Kokoska, L. (2013). Growth inhibitory effect of grape phenolics against wine spoilage yeasts and acetic acid bacteria. *Int. J. Food Microbiol*. 161, 209–213. doi: 10.1016/j.ijfoodmicro.2012.12.018

Patil, S., Bourke, P., Frias, J. M., Tiwari, B. K., and Cullen, P. J. (2009). Inactivation of *Escherichia coli* in orange juice using ozone. *Innov. Food Sci. Emerg. Technol*. 10, 551–557. doi: 10.1016/j.ifset.2009.05.011

Peixoto, C. M., Dias, M. I., Alves, M. J., Calhelha, R. C., Barros, L., Pinho, S. P., et al. (2018). Grape pomace as a source of phenolic compounds and diverse bioactive properties. *Food Chem*. 253, 132–138. doi: 10.1016/j.foodchem.2018.01.163

Riviera-Espinoza, Y., and Gallardo-Navarro, Y. (2010). Non-dairy probiotic products. *Food Microbiol.* 27, 1–1. doi: 10.1016/j.fm.2008.06.008

Sengun, I. Y. (2013). Effects of ozone wash for inactivation of S. Typhimurium and background microbiota on lettuce and parsley. *J. Food Saf.* 33, 273–281. doi: 10.1111/jfs.12050

Soto, M. L., Falqué, E., and Domínguez, H. (2015). Relevance of natural phenolics from grape and derivative products in the formulation of cosmetics. *Cosmetics* 2, 259–276. doi: 10.3390/cosmetics2030259

Syed, U. T., Brazinha, C., Crespo, J. G., and Ricardo-da-Silva, J. M. (2017). Valorisation of grape pomace: fractionation of bioactive flavan-3-ols by membrane processing. *Separat. Purific. Technol.* 172, 404–414. doi: 10.1016/j.seppur.2016.07.039

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer MC and handling Editor declared their shared affiliation.