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"Viability of 4 Wild Strains *L. paraplantarum, L. plantarum, W. paramesenteroides,* and *E. faecalis in* Fermented Probiotic Grape Marmalade during Storage at both 5°C and 25°C"

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Abstract

Grape foods fermented with probiotics are sources of beneficial bacteria for the GI tract and also have a high antioxidant capacity. The addition of probiotics to ferment food has always been a traditional process; therefore, probiotic dairy and non-dairy products might contribute to a daily antioxidant diet to improve consumers' life quality and health. This research was undertaken to determine the viability of 4 wild isolates of *Lactobacillus* for storage at 5 and 25ºC within 90 days in simulated synthetic grape media and a standard grape marmalade formulation. Changes in active culture numbers, pH level, glucose concentration, and antioxidant properties were evaluated. Most of the isolates demonstrated higher growth in the grape marmalade than the synthetic grape marmalade, which was greater than 7 Log cfu/g within 90 days of storage at 5ºC. In addition, most of the wild isolates grew beyond the critical count of 10^6 cfu/g in sampling between 60 and 90 days of storage. Moreover, fermented grape marmalade with probiotics showed a strong antioxidant capacity that failed to differ significantly with the synthetic medium. The study confirmed *L. paraplantarum, L. plantarum, W. paramesenteroides,* and *E. feacalis* were ideal probiotics for fermentation process of grape marmalade.

Keywords: Anti-oxidative properties; DPPH; Grape Marmalade**;** *Lactobacillus;* Probiotics.

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1. Introduction

Probiotics represent constituents of bacterial cells or compounds of microbial cells with beneficial aspects on human health and well-being, naturally present in fermented products such as milk, yogurt, and sauerkraut (Ouwehand and Salminen, 1998; Salminen et al., 1999). They can also be carefully selected and added to the diet according to their health benefits. However, not all microorganisms are probiotics. To earn this label, they must have proven their health benefits that they are in sufficient concentration and that they survive the acidity of the stomach to be able to act in the intestines. Most probiotics used nowadays are *Lactobacillus* and *Bifidobacterium (Vancanneyt et al., 2006)*. Lately, new specific strains and species to the Genus *Lactobacillus* have been screened as "Pro-biotic" due to the fact that health benefits conferred by bacteria seem specific to the strains. Metchnikoff, the Nobel Prize winner in 1908, discovered that the nutritional value of yoghurt was largely due to the presence of lactic acid bacteria, which does not mean that they are present in milk, but that they synthesize lactic. Since its discovery, some previous reports indicated innumerable virtues to certain strains of these bacteria: improving the digestibility of proteins, lactose, and bioavailability of minerals, and participating in the synthesis of certain vitamins, especially vitamins B and K (Parvez et al., 2006).

Certain strains of probiotics have a regulating action on the intestinal transit and can therefore be useful, both in cases of diarrhea and constipation. Some are effective against allergies or work against gastroenteritis, and others reduce inflammatory recurrence in Crohn's disease, chronic inflammation of the terminal part of the small intestine, and often of the colon (Prantera et al., 2002). Moreover, studies attribute the oral absorption of probiotics to increases in the resistance to immune diseases. Nowadays, other benefits are currently under study, including those of lactobacillus against atopic eczema or in certain types of cancer.

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Today, there is a growing demand for both non-dairy and dairy probiotic products in the market, as well as bacteria added to beverages and marketed as supplements, tablets, and freeze-dried preparations (Luckow and Delahunty, 2004). Probiotics added to a new food or beverage lead to many substantial variables to be examined to ensure survivability, which is important for safety. It should be noted that the physiological condition of the bacteria incorporated into products is of great significance, and therefore relies greatly on these 3 basic parameters; such as the time of harvest of the bacteria, whether in the midst of the log phase or stationary growth phrase; the condition resulting in static period on the handling of bacteria while and after harvesting; and the structure of the growth environment in regards to product constitution to which they may be incorporated (Ouwehand et al., 2002). Furthermore, fermented dairy and non-dairy products could include a shelf life so that they have a high viable bacteria count of at consumption, averaging a lower limit of 10^6 cfu.g-^{1} of products (De La Cruz et al., 2010). Therefore, this research aimed to investigate the viability of the probiotics added to fermented grape marmalade during storage until 90 days at room and refrigerated temperatures.

2. Materials and methods

2.1 Grape Control Media (GCM)

For the preparation of the control, a grape medium was prepared to determine the growth and survival of 4 wild strains of *W. paramesenteroides, L. paraplantarum, E. faecalis,* and *L. plantarum*. The media was formulated as followed: 55 g of sugar, 1.5 g of agar-agar, and 500 mL of condensed grape extract. Food quality Lactic Acid was added to adjust the medium to pH 4.5 as described by (Randazzo et al., 2010). The marmalade was distributed in sterile containers and maintained at 5 and 25°C before inoculated by the bacterial strains.

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2.2 Grape Marmalade Samples (GM)

The grapes used in this study were provided by a local farm in Pingtung (Taiwan) producing organic grapes (*Vitis vinifera*). The formulation of the jam followed a regular industrial recipe; 83% of grapes, 15% of sucrose, and 2% of lemon juice were mixed then heated to 40-45ºC to obtain a degree Brix (°Bx) value of 40. Then, the marmalade was stored (180 g portion) in glass jars with no addition of additives (Restuccia et al., 2006).

2.3 Bacterial strains

The 5 wild strains used in this study belong to the microbial collection of the laboratory. The isolates from local plant leaves were screened for their phenotypic, genotypic, biochemical and technological attributes. The strains have been studied previously for bacterial properties such as resistance to acid, bile, the capability to adapt to the cell line of the intestinal epithelium, antimicrobial activity, lysozyme resistance, CaCO-2 cells, and antibiotic sensitivity (Caggia et al., 2009; Scialdone et al., 2009). Thus, the *L. plantarum* strain was used as a control. All isolates were cultured in MRS and kept within 20% Glycerin at -80ºC for further use.

2.4 Preparation and inoculation of probiotic cultures into grape control media and grape marmalade

Four different isolates of probiotics isolated from 5 different plant leaves were sub-cultured in MRS medium to 1% (v / v) inoculum. Bacteria isolates were collected at 10000 rpm for 10 minutes, and maintained in a 0.9% NaCl solution, and diluted 10-fold in the same condensed solution. Then, 100 g of the grape marmalade product was aseptically dispensed into sterilized tubes (Champagne et al., 2005; Phillips et al., 2006). The products were cultured with fresh probiotic cells, as reported

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above, to a final density of 10^9 cfu/g marmalade. The bacterial solution was then mixed into the marmalade and kept at 5ºC and 25ºC. Grape marmalade saturated in salt solution (0.9% w/v NaCl) was used as control.

2.6 Physical and chemical tests of the marmalades

A pH meter was used to evaluate the pH values at regular intervals. The samples were investigated on production day and after 90 days of storage at 5 and 25ºC. To determine the sugar content of marmalade, the D-glucose Enzyme Assay Kit (K-SUFRG) (Megazyme International Ireland Ltd., Wiclkow) was used according to the manufacturer's protocols (Randazzo et al., 2013).

2.6.1- Anti-oxidative properties of probiotic grape marmalade

The anti-oxidative capability of the probiotic grape marmalade was evaluated following NO Radical, ABTS, and DPPH radical scavenging activity assays described below. The probiotic product was centrifuged at 8000 x g for 5 min at 5ºC, and the supernatants were analyzed after intervals of 0, 24, 48, and 72 h incubation to evaluate their antioxidant properties (Ruberto et al., 2007).

2.6.2- Nitric Oxide Radical (NO)

The activity of trapping plant extracts against the NO radical was assessed by the method of (Ebrahimzadeh et al., 2008). The generation of nitric oxide was measured from sodium nitroprusside by the Greiss reaction. The amount of nitrite formed was reduced between oxygen and nitric oxide generated by sodium nitroprusside. The percentage of antioxidant activity was estimated at A596 nm and calculated according to the following formula (Thaipong et al., 2006):

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AA% = [100–((A596 sample —A596 blank) × 100)] / A596 control, where A596 is absorbance at 596 nm.

2.6.3- ABTS Assay

ABTS radical scavenging activity of grape marmalade extracts was evaluated by the ABTS cation decolorization assay as described by Ruberto et al. (2001) with some modifications (Ruberto et al., 2007). The ABTS radical cation was produced by reaction of 7 mM stock solution of ABTS with 2.45 mM potassium persulfate and the mixture was allowed to stand in the dark at room temperature for 12 h before use. The ABTS solution was diluted with methanol to give an absorbance of 0.7 ± 0.01 at 734 nm. Grape marmalade extracts were allowed to react with 2 mL of the ABTS solution and the absorbance was measured at 734 nm after 1 minute. Trolox was used as a reference compound. The results were expressed as Trolox equivalent antioxidant capacity values and calculated as mean value ± standard deviation (SD) (n = 3).

2.6.4. Tests of DPPH

The procedures of Tagliazucchi were adopted to determine DPPH (Sigma-Aldrich) scavenging activity; therefore, 1 mL of grape marmalade supernatant and 5 mL newly prepared 0.1 mM DPPH methanol solution were combined and maintained in the dark for 1 hour (Tagliazucchi et al., 2010). A spectrophotometer was used to measure the absorbance at A_{517}. Thus, the grape marmalade supernatant with methanol (1 mL) was replaced by a blank to calculate the proportion of DPPH as follows:

DPPH (%) = [1 - (A_{517} nm sample/A_{517} nm blank)] x 100%, where A_{517} is absorbance at 517 nm.

2.7 Enumeration of probiotic bacteria

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Aliquots of both control and GM samples, inoculated and non-inoculated from the original isolates, were evaluated at regular intervals of 0, 15, 30, 45 and 90 days at 5 and 25℃ of storage, for enumeration of viable bacteria according to the procedure of (Randazzo et al., 2013).

2.8 Statistical analysis

All data were submitted to ANOVA for statistical analysis. Then, the data were compared via the statistical procedure of SPSS and the Tukey test ($p < 0.05$).

3. Results

3.1. Physicochemical parameters of grape Control media and marmalade samples

In Table 1, the pH values in GCM of inoculated and non-inoculated samples are shown with the 4 isolates at both room and refrigerated storages. Overall, reduction in pH of all samples persisted at 5℃ of storage. Interestingly, the inoculated GCM samples with wild-type strains exhibited identical pH values at 25°C to other reports. The synthetic medium inoculated with selected isolates indicated particularly a substantial reduction at 25°C of storage attaining pH 3 to 3.4 within 60 days. Thus, a distinct trend of pH was noticed in the synthetic medium to wild-type isolates at refrigerated conditions. The grape synthetic medium samplings inoculated with isolates *W. paramesenteroides, E. faecalis,* and *L. plantarum* exhibited significant pH values during storage; however, the synthetic medium inoculated with the strain *L. paraplantarum* had fairly regular pH levels.

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Table 1. pH values about LAB counts for the non-inoculated and inoculated GCM and GM kept at room and refrigerated temperatures.

pH values in Grape Control Media (GCM)

Time (days)		Control	*L. plantarum*	*E. faecalis*	*L. paraplantarum*	*W. paramesenteroides*
25°C	0	4.10±0.03a	4.12±0.08a	4.03±0.08a	3.97±0.12a	4.13±0.13a
	15	3.82±0.04a	3.77±0.10ab	3.70±0.07ab	3.73±0.07a	3.69±0.09ab
	30	3.79±0.06a	3.75±0.04a	3.72±0.04a	3.71±0.09a	3.69±0.05ab
	60	3.70±0.08ab	3.64±0.06b	3.67±0.13ab	3.64±0.09b	3.72±0.02a
	90	3.52±0.09ab	3.47±0.05b	3.52±0.11b	3.49±0.04b	3.45±0.08b
4°C	0	4.08±0.10a	4.05±0.08a	4.02±0.11a	3.98±0.09ab	4.07±0.13a
	15	3.81±0.07a	3.79±0.04a	3.75±0.14a	3.81±0.04a	3.85±0.14a
	30	3.71±0.06ab	3.68±0.16ab	3.70±0.06ab	3.73±0.06a	3.74±0.06a
	60	3.73±0.08ab	3.69±0.08ab	3.66±0.08ab	3.70±0.09a	3.70±0.08a
	90	3.54±0.09b	3.53±0.07ab	3.54±0.09b	3.50±0.07ab	3.52±0.09b

Formulation of values as the average ±S.D. in triplicate runs. Same columns by different letters indicated significant differences with a *p-value* greater than 0.05.

pH values in Grape Marmalade (GM)

Time (days)		Control	*L. plantarum*	*E. faecalis*	*L. paraplantarum*	*W. paramesenteroides*
25°C	0	4.08±0.12a	4.05±0.10a	3.91±0.14a	3.85±0.10a	3.78±0.17a
	15	3.83±0.07ab	3.76±0.04a	3.77±0.24ab	3.71±0.17a	3.68±0.16ab
	30	3.79±0.16a	3.75±0.16a	3.72±0.16ab	3.70±0.06a	3.69±0.16ab
	60	3.75±0.08ab	3.68±0.18ab	3.67±0.08ab	3.70±0.14a	3.67±0.18ab
	90	3.53±0.12ab	3.49±0.09b	3.51±0.12b	3.49±0.09ab	3.58±0.09b
4°C	0	4.07±0.09a	4.02±0.18a	3.97±0.13a	3.95±0.08a	3.90±0.11a
	15	3.85±0.34a	3.80±0.24a	3.77±0.31a	3.81±0.17a	3.81±0.23a
	30	3.71±0.25a	3.71±0.16ab	3.69±0.21ab	3.71±0.18a	3.70±0.21a
	60	3.68±0.17ab	3.65±0.17a	3.63±0.14ab	3.69±0.41a	3.69±0.18a
	90	3.55±0.29b	3.52±0.15b	3.52±0.18ab	3.51±0.12ab	3.52±0.13ab

Formulation of values as the average ±S.D. in triplicate runs. Same columns by different letters indicated significant differences with a *p-value* greater than 0.05.

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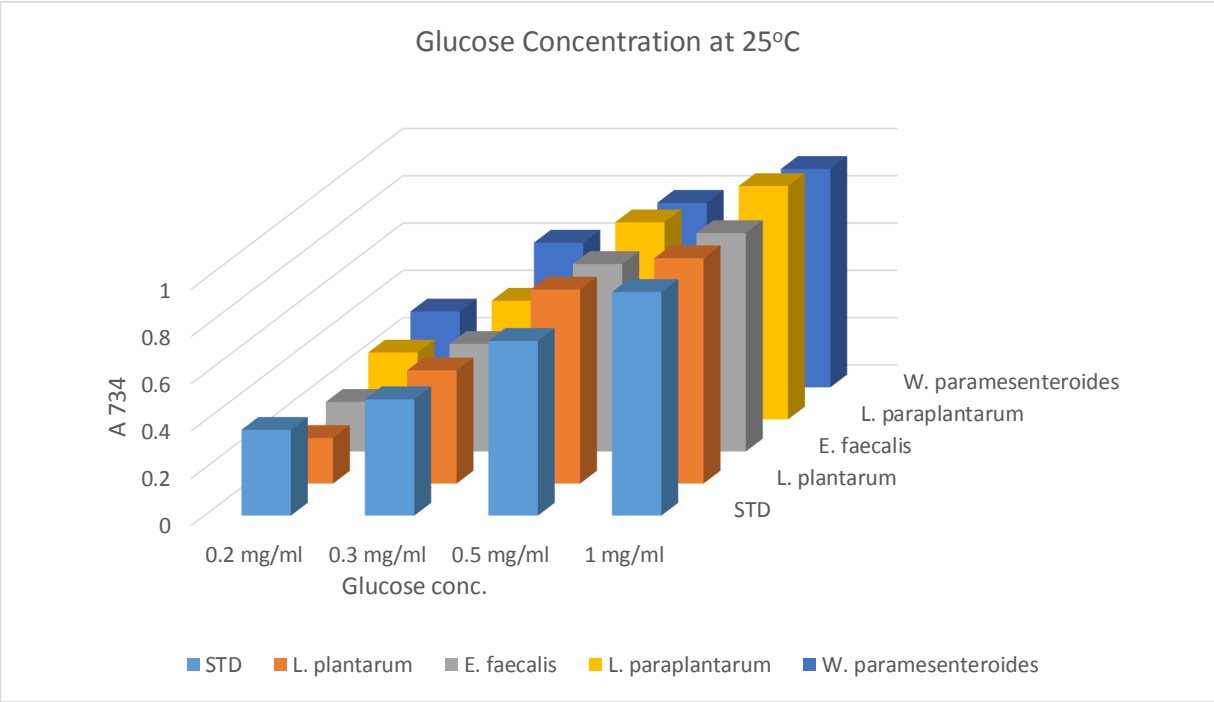
The sugar concentration in grape marmalade was evaluated to 100 mg/g of Glucose until the end of preservation. The results of non-inoculated and inoculated grape marmalades were shown in Fig. 1 and sugar composition was 0.339 g of Glucose at A_{534} for the freshly prepared samples. Jams and marmalades are basically constituted of fruit peel, sugar, and water; in addition, the more bitter the peel the better the flavor of the marmalade. It represents a beneficial origin of dietary fibers, vitamins, and minerals. These nutrients possess healthy benefits in several ways considering when they are incorporated with probiotic bacteria and antioxidants.



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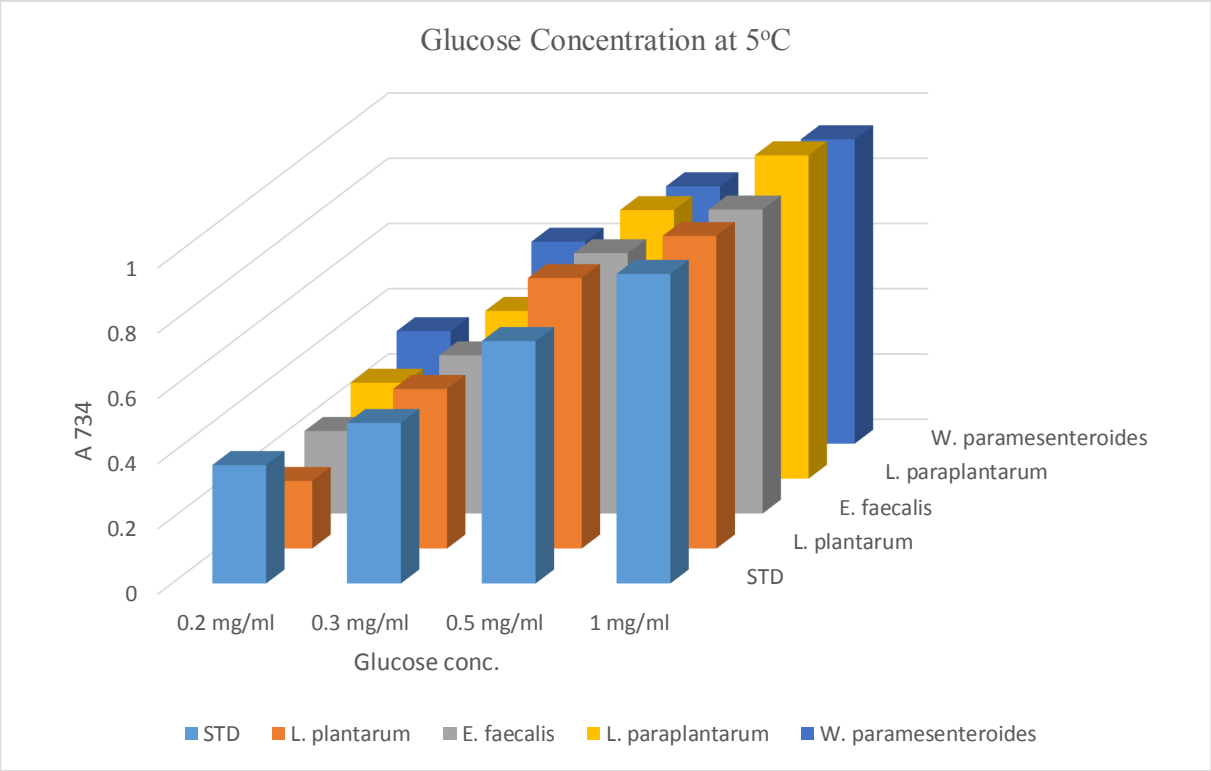

Figure 1: Glucose Concentration of the Marmalade at different Temperatures of Storage 5ºC and 25ºC

3.2. Viability of probiotic Bacteria in GCM and GM at storage

The viability of probiotic strains at storage was investigated in the synthetic medium and marmalade of grapes at refrigerated and room temperatures (Table 2). Samples of non-inoculated grape synthetic medium and marmalade exhibited slight numbers in the media during preservation at room and refrigerated conditions. Generally, synthetic medium samples inoculated with the wild-type *W. paramesenteroides, E. faecalis,* and *L. plantarum* indicated strong growth within 30 days of storage at room temperature, whereas specimens inoculated to *L. paraplantarum* showed seemingly constant values (Table 2). All synthetic medium samples indicated a substantial reduction in survival over the crucial range of 10^6 cfu/g for more than 45 days. Nevertheless, a distinct tendency was noticed at 5°C: most initial isolates showed a reduction after 30 days (log 8

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cfu/mL) of preservation and growth within 45 days. Strains of *W. paramesenteroides, E. faecalis,* and *L. plantarum* remained viable in the grape synthetic medium during 90 days of storage, yet subsisting beyond the crucial counts of 10^6 cfu/g. Most of the initial isolates exhibited strong survival with considerable growth in 30 days at room temperature, according to cells density ranging from 10^{10} to 10^{12} cfu/g. Most isolates excluding *E. faecalis* had counts over the crucial standard of 10^6 cfu/g for more than 60 days at 25°C (Table 2). Thus, most of the isolates stayed active above 10^7 cfu/g in the grape marmalade samplings kept at refrigerated condition. Under those terms, all initial isolates indicated significant counts (proximate to 10^{10} cfu/g) from 15 to 45 days of storage, excluding *W. paramesenteroides* and *E. faecalis,* which maintained the greatest values after 60 days of storage.

Table 2. LAB counts (Log cfu/g) for the non-inoculated and inoculated GCM and GM kept at room and refrigerated temperatures.

Mean counts (Log cfu/g) Grape Control Media (GCM)

Time (days)		Control	*L. plantarum*	*E. faecalis*	*L. paraplantarum*	*W. paramesenteroides*
25°C	**0**	8.90±0.13a	8.73±0.27a	8.81±0.18a	8.75±0.23a	8.84±0.21a
	15	9.22±0.74a	9.01±0.69a	9.19±0.71a	9.26±0.72a	9.89±0.68a
	30	8.75±0.86a	7.98±0.23a	6.75±0.37a	8.67±0.89a	8.42±0.85a
	60	8.72±0.48a	6.25±0.51b	4.98±0.63c	6.28±0.49b	7.21±0.52a
	90	7.44±0.29a	5.71±0.23c	3.74±0.21c	5.74±0.54b	5.77±0.18b
4°C	**0**	8.92±0.24a	8.77±0.26a	8.85±0.21a	8.98±0.28a	8.72±0.17a
	15	9.26±0.73a	9.17±0.71a	9.09±0.70a	9.19±0.68a	8.53±0.74a
	30	8.80±0.93a	8.38±0.86a	8.61±0.91a	8.68±0.87a	8.75±0.91a
	60	8.68±0.62a	7.41±0.49a	9.02±0.54a	8.08±0.51a	9.01±0.59a
	90	7.48±0.57a	6.17±0.35b	7.51±0.56a	7.50±0.60a	6.77±0.11a

Formulation of values as the average ±S.D. in triplicate runs. Same columns by different letters indicated significant differences with a *p-value* greater than 0.05.

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Mean counts (Log cfu/g) Grape Marmalade (GM)

Time (days)		Control	*L. plantarum*	*E. faecalis*	*L. paraplantarum*	*W. paramesenteroides*
25°C	0	8.92±014a	8.70±0.24a	8.79±0.21a	9.02±0.69a	8.81±0.45a
	15	9.13±0.82a	9.07±0.75a	9.04±0.77a	8.86±0.67a	9.06±0.71a
	30	8.78±0.50a	8.75±0.59a	7.80±0.53a	8.71±0.82a	8.71±0.46a
	60	9.08±0.61a	8.98±0.56a	8.88±0.59a	8.75±0.67a	8.95±0.89a
	90	6.84±0.53a	7.17±0.48a	6.84±0.52a	6.77±0.12a	7.73±0.36a
4°C	0	8.93±0.38a	8.77±0.66a	8.78±0.37a	9.10±0.84a	8.86±0.3a
	15	9.16±0.85a	9.29±0.92a	9.19±0.89a	9.26±0.98a	9.14±0.81a
	30	8.78±0.34a	8.83±0.78a	8.75±0.41a	8.85±0.79a	8.85±0.33a
	60	8.75±0.28a	9.11±0.96a	8.95±0.19a	9.15±0.96a	9.11±0.93a
	90	7.46±0.19a	8.74±0.58a	7.84±0.30a	8.86±0.87a	7.81±0.13a

Formulation of values as the average ±S.D. in triplicate runs. Same columns by different letters indicated significant differences with a *p-value* greater than 0.05.

3.3. Correlation and Properties of the fermentation

The isolates of *L. paraplantarum, W. paramesenteroides, E. faecalis,* and *L. plantarum* performed well in sterile grape marmalade with no addition of nutrients. Tables 1 and 2 summarizes the evolution of lactic fermentation time of grape marmalade by *L. paraplantarum, W. paramesenteroides, E. faecalis,* and also *L. plantarum*, respectively. The counts of *L. plantarum, W. paramesenteroides,* and *E. faecalis* attained 10^9 cfu/mL in grape marmalade after 3 days of fermentation at 30°C (Table 2). The extension of the development time beyond 15 days showed no significant decrease in the cell counts of all the tested LAB. Under fermentation of grape marmalade, sugar metabolism was described by glucose absorption, but fermented grape marmalade maintained sugars at the acidification outset. Both *L. plantarum* and *W. paramesenteroides* generated more lactic acid than *L. paraplantarum*. In reference, *L. plantarum* and *W. paramesenteroides* generated about 1% of lactic acid after 3 days at 30°C. In identical development terms, *L. paraplantarum* generated a titratable acidity of 0.79% acid lactic. Thus, only *L. paraplantarum* failed to significantly correlate with other strains at 5°C for the counts in

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234 the grape marmalade (Table 3); but most isolates showed a certain positive correlation at some
235 points with a *p-value* = 0.01 or *p-value* = 0.05. Nonetheless, a different case is observed for the
236 acidity of the strains at room and refrigerated conditions. At *p-values* 0.01 and 0.05, none of the
237 isolates showed significant differences (Table 4), since *E. faecalis* and *L. plantarum* exhibited
238 significant correlation of the strains at 25°C and 5°C.

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239 Table 3: Pearson's correlation coefficients of *Lactobacillus* counts in the Grape Marmalade at both 25°C and 5°C.

		Control	*L. plantarum*	*E. faecalis*	*L. paraplantarum*	*W. paramesenteroides*	*Control*	*L. plantarum*	*E. faecalis*	*L. paraplantarum*	*W. paramesenteroides*
	Control	1									
	L. plantarum	.995**	1								
25°C	*E. faecalis*	.913*	.900*	1							
	L. paraplantarum	.984**	.965**	.882*	1						
	W. paramesenteroides	.993**	.995**	.938*	.964**	1					
	Control	.978**	.972**	.898*	.978**	.977**	1				
	L. plantarum	.588	.646	.691	.453	.672	.581	1			
5°C	*E. faecalis*	.974**	.985**	.927*	.932*	.992**	.972**	.743	1		
	L. paraplantarum	.672	.673	.902*	.606	.740	.681	.811	.765	1	
	W. paramesenteroides	.992**	.998**	.919*	.954*	.997**	.963**	.678	.988**	.711	1

** It means the values are significantly correlated at *p-value* = 0.01

* It means the values are significantly correlated at *p-value* = 0.05

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244 Table 4: Pearson's correlation coefficients of the pH values in the Grape Marmalade at both 25ºC and 5°C.

		Control	*L. plantarum*	*E. faecalis*	*L. paraplantarum*	*W. paramesenteroides*	Control	*L. plantarum*	*E. faecalis*	*L. paraplantarum*	*W. paramesenteroides*
	Control	1									
	L. plantarum	.996**	1								
25°C	*E. faecalis*	.991**	.990**	1							
	L. paraplantarum	.976**	.959**	.940*	1						
	W. paramesenteroides	.991**	.990**	.966**	.984**	1					
	Control	.985**	.991**	.997**	.925*	.962**	1				
	L. plantarum	.983**	.984**	.999**	.924*	.954*	.997**	1			
5°C	*E. faecalis*	.984**	.988**	.999**	.925*	.958*	.999**	.999**	1		
	L. paraplantarum	.985**	.971**	.987**	.961**	.959**	.973**	.983**	.979**	1	
	W. paramesenteroides	.968**	.947*	.970**	.953*	.938*	.950*	.966**	.959**	.996**	1

** It means the values are significantly correlated at *p-value* = 0.01

* It means the values are significantly correlated at *p-value* = 0.05

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3.4. Antioxidant characteristics of probiotic grape marmalade

The antioxidant influence of LAB, in terms of the antioxidant capability of cells and the intracellular cell-free extract of LAB, has been investigated based on several anti-oxidative tests. In this research, the anti-oxidative activity of grape marmalade is shown with various cultures of *L. plantarum, E. faecalis, W. paramesenteroides,* and *L. paraplantarum* (Table 5). In particular, according to the cultures applied, the fermented grape marmalade exhibited a strong antioxidant capability, estimated as total anti-oxidative activity, ABTS, and DPPH. Nonetheless, the probiotic grape marmalade that contained *W. paramesenteroides* indicated an anti-oxidative activity of 77% that varied insignificantly from that of probiotic grape marmalade with *L. paraplantarum* and *L. plantarum* (72% and 71%, respectively) within 3 days of fermentation.

Anti-oxidative activity decreased with *E. faecalis* after fermentation (15.3% versus 8.7%), regarding a decrease of 7% over 90 days. An apparent decrease in antioxidative activity was observed in the grape marmalade using *L. paraplantarum* (decreased about 5%). The ABTS in the probiotic grape marmalade with LAB exhibited no significant differences during storage at room and refrigerated conditions. GM fermented with *E. faecalis* and *W. paramesenteroides* exhibited higher ABTS (69%) than those of *L. plantarum* and *L. paraplantarum*, which inhibiting powers were 67.2 and 64.7 respectively. Similar findings were obtained for DPPH, where the probiotic grape marmalade with *W. paramesenteroides* exhibited the highest scavenging activity (73%). DPPH capacity of grape marmalade was decreased, notably of the probiotic *L. paraplantarum*, after 90 days of storage at room temperature.

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272 Table 5: Anti-oxidative Properties of the Grape Marmalade (% inhibition)

	NO Radical (%-A=534 nm)				ABTS (%-A=700 nm)				DPPH (%-A=340 nm)			
Time (day)	*L. plantarum*	*E. Faecalis*	*L. para plantarum*	*W. para-mesenteroides*	*L. plantarum*	*E. faecalis*	*L. para-plantarum*	*W. para mesenteroides*	*L. plantarum*	*E. faecalis*	*L. paraplantarum*	*W. para-mesenteroides*
0	15.3±0.8a	14.4±0.9a	13.1±0.9a	14.9±1.1a	67.2±2.4a	69.2±1.6a	64.7±1.3a	69.8±1.2a	72.9±3.1a	73.1±3.4a	71.4±2.5a	73.4±3.1a
15	16.6±0.5a	13.7±0.7a	14.5±0.8a	16.3±0.9a	65.5±1.9a	67.5±1.5a	62.5±1.4a	68.5±1.1a	72.1±2.9a	72.8±3.2a	70.7±1.9a	72.7±3.0a
30	14.8±0.7a	13.6±0.7a	12.1±0.8a	14.4±0.8a	63.3±1.6a	64.6±1.5a	59.6±1.3b	67.2±1.2a	70.4±3.2a	71.9±2.3a	68.7±1.3a	70.6±2.3a
45	13.7±0.4a	11.8±0.6a	11.9±0.7a	12.8±0.7a	62.4±1.7a	62.4±1.6a	57.2±1.4b	64.3±1.1a	68.8±1.2a	69.9±1.8a	67.3±1.1a	68.8±1.8a
60	12.9±0.4a	12.9±0.7a	10.4±0.8b	11.7±0.8a	59.7±1.1b	60.9±1.4b	55.4±1.2c	62.7±1.3a	66.9±1.8a	67.4±1.6a	65.8±1.5b	67.9±2.1a
90	8.7±0.3b	8.7±0.5b	8.6±0.6b	9.8±0.6b	54.2±1.2c	58.7±1.3b	53.5±1.1c	59.4±1.2b	64.7±1.9b	65.3±1.8b	63.6±1.3b	66.7±2.1a
GSM	14.8±0.5a				66.3±2.2a				71.8±2.2a			

273 Formulation of values as the average ±S.D. in triplicate runs. Values with similar letters showed non-significant differences with a *p-value* greater than 0.05.

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4. Discussion

The cholesterol content and lactose non-tolerance reside in two substantial challenges combined with milk-based products. In addition, there is an increase for vegetarianism in some developed countries and the demand for non-dairy probiotic products (Kumar et al., 2015). Numerous studies have recently shown that certain raw materials are capable of introducing new non-dairy probiotic products into the market (Abu-Ghannam and Rajauria, 2015; Rivera-Espinoza and Gallardo-Navarro, 2010). Thus, a proper study of non-dairy probiotic products is emphasized on the numerous fundamental and external properties of foods, such as pH, nutrient accessibility, glucose content, O_2 level, water, and temperature, affect the survival of probiotic cells (Mårtensson et al., 2002; Mattila-Sandholm et al., 2002; Vasudha and Mishra, 2013). In this research, the survival of four different wild strains was studied in grape marmalade at room and refrigerated conditions of storage. All the isolates were investigated for their characteristics, viability to pH 2, strong tolerance to bile salts, resistance to different "Anti-biotics", such as vancomycin, chloramphenicol, tetracycline, ampicillin, and penicillin, and their antibacterial trend against *S. aureus, L. monocytogenes*, and *E. coli*. This study was able to show that the alleged wild probiotic strains survived in the grape marmalade during storage, thus indicating that this vehicle might be a potential candidate as a probiotic medium. Thus, the isolates exhibited significant viability more in grape marmalade than in the synthetic medium grape, which confirmed that food preparations could affect probiotics survival at storage. Some authors have argued that a strong matrix can preserve the strains during storage; thus in this research, the grape marmalade preparation with its innate constituents, apparently promoted the survival of the probiotics (Lacroix and Yildirim, 2007; Ranadheera et al., 2010; Saarela et al., 2000). Previous researches have demonstrated the viability of probiotic strains in highly acid food matrix during storage (4-5°C), claiming that the

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298 increase and survival of cells in some fruits and vegetables may be subject to the strains applied
299 (Chen and Chen, 2007; Tuomola et al., 2001). In this research, the survival of the strains confirmed
300 the results shown by previous studies, which showed the probiotic strain cells had a comparative
301 stability in milk compared to non-dairy products.
302 The changes in the stability of isolates were attributed to pH and Temperatures during storage.
303 Champagne reported that in several fermented milk byproducts, the deficit of probiotic strains
304 survival was assigned to a reduction of pH (pH 4-5) and to the production of organic acids as a
305 result of fermentation (Champagne et al., 2005). Sheehan indicated that the isolates demonstrated
306 strong survival at pH 4, and indicated that the control strain might survive during 12 weeks at 5°C
307 in orange juice (pH 3.65) and in pineapple juice at pH 3.4 (Sheehan et al., 2007). This research
308 reported that most of the isolates stayed active beyond the crucial count in grape marmalade at
309 room temperature within 45 days. The correlation in bacteria numbers as well as pH levels
310 confirmed the survival of isolates, related to previous statistics, while the survival of the wild
311 strains was to a lesser degree. In addition, the results indicated an insignificant increase in glucose
312 concentrations at 25°C of storage. The increase in glucose might be due to two factors that occurred
313 concurrently: intracellular bacterial growth and sucrose inversion, which was precipitated at low
314 pH levels and at high storage temperatures. Essentially, marmalades and jams are considered as
315 high stable products referring to their low pH, glucose concentration, and their A_w; they constitute
316 suitable media to sucrose inversion (Agte et al., 2010; Simanjuntak et al., 2013).
317 This study also highlighted the anti-oxidative effect of Lactic Acid Bacteria as a great interest for
318 researchers (Chu et al., 2000; Wang et al., 2008). The anti-oxidative effect of grape marmalade is
319 presented in Table 2, using different strains of *L. plantarum, W. parmesenteroides, E. faecalis*, and

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320 *L. paraplantarum*. Based on the strains applied, the grape marmalade demonstrated strong
321 antioxidant capacity, evaluated as DPPH scavenging activity, and total antioxidant activity.
322 Similar findings were exhibited for DPPH: grape marmalade fermented with *L. plantarum* and *W.*
323 *parameseneroides* showed the highest scavenging activity at 70%; whereas *L. paraplantarum*
324 reduced the DPPH of grape marmalade within 30 days at room temperature. In a study conducted
325 by Wang *et al*. (2008), it was observed that the fermentation of carbohydrates by innate intestinal
326 LAB exhibited strong antioxidant effects. Table 2 indicated that the glucose concentration of the
327 grape marmalade was probably a sufficient and essential carbon source for Lactobacilli strains
328 (Chen et al., 2008).
329 The metabolism of the bacteria apparently affected the capacity of the antioxidant activity in the
330 marmalade according to the strains used. Some researchers have reported the effect of fermentation
331 on the food anti-oxidative properties. For instance, Espinoza *et al*. (2010) reported the anti-
332 oxidative characteristics of fermented probiotic carrot juice (Rivera-Espinoza and Gallardo-
333 Navarro, 2010); and it's reported that sugar apple as substrate indicated no significant difference
334 between fresh and fermented juices for the strains of *L. delbrueckii, L. paracasei*, *and L. casei.*
335 Fermented sugar apple juice showed 65%-75% antioxidant activity and DPPH scavenging free
336 radicals scavenging of 72% (Prado et al., 2008). The fermented grape marmalade showed anti-
337 oxidative activities, which changed with the strains applied, but *L. plantarum* and *L.*
338 *paraplantarum* had no effect on the anti-oxidative activity of fermented grape marmalade
339 compared to non-fermented synthetic medium. In this study, the fermentation of grape marmalade
340 with *W. paramesenteroides* showed better anti-oxidative activity than the non-fermented synthetic
341 medium (Table 5). As a result, the anti-oxidative activity changed with the starters used.
342 Furthermore, the accumulation of intracellular sucrose in response to osmotic stress may explain

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the reduction of sucrose; however, this assumption needs to be studied in more detail (Randazzo et al., 2013). The findings of this study showed that *L. plantarum* and *W. paramesenteroides* were the best probiotic bacteria to produce non-dairy probiotic products. Moreover, antioxidant ability exhibited for 72 hours of fermentation period by *E. faecalis* showed no significant difference comparing to *L. paraplantarum, L. plantarum,* and *W. paramesenteroides*. Grape marmalade might be a good candidate for the production of new functional probiotic, which might efficiently subsist probiotic strains of *Lactobacillus* both under refrigerated conditions and at room temperature. A quotidian absorption of approximately 10 grams of probiotic marmalade might provide from 10^8 to 10^9 viable probiotic bacteria, with stored preparations for 40 - 90 days at both 25ºC and 5ºC, respectively (Ouwehand et al., 2002). Similarly, the results of this study are assimilated to those in fermented dairy products, with over to 10^6 cfu/mL within 30 days of storage at 5ºC.

5. Conclusions

The capability of *L. paraplantarum, L. plantarum, W. paramesenteroides,* and *E. faecalis* was investigated in the suitability of grape as a raw material for the production of probiotic grape marmalade during storage. All wild strains of Lactobacillus showed better viability at different temperatures of storage compared to the control isolates, with a greater count by log 7 cfu/g up to 90 days and stored at 5°C. Grape marmalade isolates, stored at room temperature, maintained sustainable counts beyond the crucial level for 90 days. The metabolism of lactobacilli caused pH changes that accelerated the rate of hydrolysis of sucrose to constituent glucose. Based on a commercial aspect, the viability might be considered as an important criterion for the selection of isolates at different temperatures for use in probiotic marmalades during storage.

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Conflict of Interest

The authors declare no conflict of interest.

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