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# Antioxidant and Anthocyanin Content in Fermented Milks with Sweet Cherry is Affected by the Starter Culture and the Ripening Stage of the Cherry

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**Abstract:** Fermented milk-based beverages containing fruits are perceived as healthy foods by consumers and are increasingly demanded. The incorporation of sweet cherry into fermented milks was evaluated in the present study. Maturation stage of cherry 8 and 12 (commercial and over-ripened) and starter culture (*Lactobacillus casei*, *Lactobacillus paracasei* and *Lactobacillus helveticus*) were tested. Antioxidant properties, anthocyanin content, color and microbial counts were used to assess the quality of the fermented milks. *L. helveticus* exhibited the fastest acidification rate; whereas *L. casei* and *L. paracasei* presented the highest microbial counts. Fermented milks containing grade 12 sweet cherries yielded the highest concentration of anthocyanins and color intensity and preserved phenolic compounds and anthocyanins during 10 days of refrigerated storage. *L. helveticus* preserved the highest content of phenols, whereas *L. casei* and *L. paracasei* better preserved anthocyanins. Overall, fermented milk with *L. helveticus* including grade 12 cherry puree provided the best preservation of bioactive compounds.

**Keywords:** *Prunus avium*; phenolics; *Lactobacillus*; antioxidant capacity

## 1. Introduction

Sweet cherry (*Prunus avium*) is native to Europe and western Asia [1] and it has a high commercial relevance in Spain; the main factors driving consumer acceptance of cherry are sweetness, acidity and color. For this reason, a series of parameters have been used to establish the optimal harvest time, with the color of the skin being the most reliable. The development of the red color, typical of cherry, is used as an indicator of quality and ripening, and it is due to the accumulation and profile of anthocyanins [2]. From the point of view of cherry quality, the color is fundamental to maintain a natural appearance of the fruit. However, color can be altered during storage by the action of light, temperature, oxygen, metal ions and enzymes [3]. Another relevant quality factor is firmness, because an excessive softening of the fruit is one of the main factors responsible for the limited shelf life of cherries during storage and commercialization. Consumers prefer firm fruits. Accelerated softening also contributes to an increase in the physical damage during handling, and increases susceptibility to pests and diseases [4].

Cherries can be considered a healthy food, due to their high content of polyphenols and anthocyanins. Cherries contain an estimated 160–170 mg of total polyphenols in a 100 g serving; however, no recommended daily intake of polyphenols has been yet established. Thus, it is not possible

at the moment to estimate the optimal dose of cherries in terms of health effects [1]. The phenolic compounds contribute to the quality of the fruit and to the nutritional value in terms of color development, flavor, and aroma and also may provide beneficial health effects [5].

Anthocyanins represent the main water-soluble pigments visible to the human eye, providing red, purple and blue colorations to many fruits, vegetables and cereal grains. They belong to the flavonoid group and their basic structure is a flavon nucleus, which consists of two aromatic rings joined by a unit of three carbons. The glycosides of the anthocyanidins (aglycon) are called anthocyanins, but the term anthocyanin is commonly used to encompass both the glycosylated form and the anthocyanidin itself.

Anthocyanin content and antioxidant activity are related to the ripening stage and variety of the cherry [6]. However, more studies are needed to establish the real implications of anthocyanins in these health-promoting properties [7]. Cherries are highly demanded seasonal fruits, with a limited shelf life; cherry containing foods are also highly valued by consumers. Fermented milks are also perceived as healthy foods, and the development of fermented milks containing cherry puree and with no sugar addition may provide a food valued by health driven consumers. In this sense, it is necessary to evaluate the effect of fermentation on the most valued cherry characteristics: antioxidant properties, anthocyanin content and color, and the microbial counts of lactic acid bacteria. In previous studies, it has been reported that color preservation and anthocyanins in fruit containing fermented milks are highly dependent on the bacterial strain responsible of fermentation [8,9].

*Lactobacillus* species are important ingredients in many traditional food products, especially in dairy products, and a large part of the attention has been directed towards their potential role as probiotics [10,11]. Strains that have been examined for their probiotic effects include: *Lactobacillus acidophilus* LA1, *Lactobacillus acidophilus* NCFB 1748, *Lactobacillus rhamnisus* GG, *Lactobacillus casei* Shirota, *Lactobacillus gasseri* ADH and *Lactobacillus reuteri* [10].

There is a diversity of metabolic properties associated to members of the genus *Lactobacillus* present in fermented food products. They contribute to preservation, nutritional availability and taste. Many dairy products are fermented by *Lactobacillus* [10].

The health effects attributed to the consumption of probiotic *Lactobacillus* include immunological improvement, the reduction of fecal enzyme activity, the prevention of intestinal disorders, and the reduction of viral diarrhea. Most strains of probiotics are thought to have the ability to colonize the intestinal tract and thereby have a positive effect on the microflora, preventing colonization by pathogens [10]. Likewise, *L. casei* Shirota ATCC 27092 is used in the industrial production of Yakult, and it is known for its therapeutic effects [12].

Average consumption of fermented milks in Spain was 15.34 L/person/year in 2016, growing 0.6% in relation to the previous year (includes yogurt, yogurt with bifidus and other fermented milk from lactic acid cultures: *Lactobacillus*, *Lactococcus*, and *Leuconostoc*). The purchase of this category accounted for 2.28% of expenditure on food and beverages in Spanish households in 2016 [13]. Fermented milks containing fruit are highly appreciated by consumers; they are usually sweetened and contain preserved fruit. However, little or no information is available about fermented milks with the addition of fresh fruit at different ripening stages and no sugar addition on the effect of fermentation, the anthocyanin content and antioxidant properties of the fermented milks. Highly perishable fruits such as cherries may yield noncommercial fruits at different stages of ripening, depending on pre-harvest damage (under-ripened fruits) or post-harvest (ripened or over-ripened), that maybe used to prepare fruit purees rich in bioactive compounds to be used by the food industry. In the case of cherries that may allow the use of over-ripened cherries that may not be suitable to be marketed as fresh fruits.

Thus, the aim of the present study was to evaluate antioxidant properties, anthocyanin content, phenolic compounds, color and microbial counts of fermented milks with the incorporation of sweet cherry at two maturation stages of cherry (commercial and over ripening) to determine starter cultures and ripening stage suitability.

## 2. Materials and Methods

### 2.1. Plant Material

Sweet cherries of ‘Sweet heart’ cultivar, were collected from “Fincas Toli S.L.” located at Jumilla (38.473800 N, −1.323861 W, Murcia, Spain) at two different ripening stages: 8 and 12, as described by Serrano et al. [5] in which sweet cherries were grouped in 14 different stages, being the highest value in the scale the most advanced ripening stage. Sweet cherries were washed in chlorinated water 100 ppm, rinsed twice and allowed to dry. Clean cherries were crushed under extremely hygienic conditions, and then vacuum packed, frozen and kept frozen until use at a −40 °C freezer. Microbial counts on cherry puree were determined on Plate Count Agar at 35 °C for 48 h, molds and yeasts were counted on Potato Dextrose Agar (PDA) at 26 °C for 5 days.

### 2.2. Starters

Starter cultures were lactic acid bacteria (LAB) individually used for fermented milk production: (S1) *Lactobacillus casei* CECT 475; (S2) *Lactobacillus paracasei* subs. *paracasei* CECT 277; (S3) *Lactobacillus helveticus* CECT 541 (CECT: Colección Española de Cultivos Tipo-Spanish Culture Type Collection-, Universidad de Valencia, Burjasot, España). Strains were selected based on their potential probiotic activity and previous use in fruit enriched fermented milks [8]. The inoculum was prepared from lyophilized cultures following instructions by CECT: activated in MRS broth for 24 h at 37 °C, and the second active pass was used to inoculate milk.

### 2.3. Fermented Milk Preparation and Cherry Addition

Skim milk powder (SMP) (Central Lechera Asturiana, CAPSA, Granada-Siero, Spain) was reconstituted with deionized water, which is a common industrial practice [14] at 10% *w/v* total solids to serve as control (C). SMP was reconstituted at 15% *w/v* total solids to be further completed with sweet cherry puree to have a final milk solid content of 10% *w/v* after fruit addition. Reconstituted skim milk (RSM) was pasteurized into 1-L Pyrex flasks (at 80 °C for 30 min) followed by cooling by immersion in a water bath until 43 °C were reached. After cooling, sweet cherry puree, from fruits at ripening stages 8 and 12, were added to the 15% RSM to an end concentration of 15% *w/v* of cherry (8RS and 12RS). At this point, C, 8RS and 12RS milks were distributed into 60 mL sterile flasks. A total of 30 flasks were obtained from each type of milk, so 10 flasks from each type of milk were inoculated with each starter culture. The inoculated milks were incubated at recommended growth conditions (30 °C *L. paracasei*, 37 °C *L. casei* and *L. helveticus*) for each culture, and, then, cooled down to 4 °C and stored for up to 10 days. Milks were sampled for analysis at time 0 (before incubation), 1 day of refrigerated storage of the fermented milks, and after 10 days of refrigerated storage. Three independent replicates of the whole experiment were run.

### 2.4. Plant Material Analysis

#### 2.4.1. Total Phenolics

Total phenolics were extracted according to the protocol by Tomás-Barberán et al. [15] using water/methanol (2:8) containing 2 mM NaF (to inactivate polyphenol oxidase activity and prevent phenolic degradation) and quantified in duplicate using the Folin–Ciocalteu reagent, and results (mean ± SE) were expressed as mg gallic acid equivalent per 100 g of fresh weight (fw).

#### 2.4.2. Anthocyanin Determination

Anthocyanin content was determined by the method described by Martínez-Esplá et al. [16] and was adapted to cherry tissue. Two grams of fruit tissue were homogenized in 4 mL of methanol and left for 1 h at −18 °C. Extracts were centrifuged at 15,000 rpm for 15 min at 4 °C. The supernatant was loaded onto a C18 Sep-Pak cartridge, previously conditioned with 5 mL of methanol, 5 mL of pure

water and then with 5 mL of 0.01 N HCl. The cartridge was washed with 5 mL of pure water and, then, eluted with acidified methanol MeOH (0.01% HCl). Absorbance of the collected fraction was measured at 530 nm. Total anthocyanin content was calculated using cyaniding-3-glucoside (molar absorption coefficient of  $23,900 \text{ L cm}^{-1} \text{ mol}^{-1}$  and molecular weight of  $449.2 \text{ g mol}^{-1}$ ), and results were expressed as  $\text{mg } 100 \text{ g}^{-1} \text{ fw}$ .

#### 2.4.3. Total Antioxidant Activity

Total antioxidant activity (TAA) was quantified in duplicated in each sample as previously described by Martínez-Esplá et al. [16]. Briefly, 2 g puree were homogenized in 15 mL of 50 mM Na-phosphate buffer pH = 7.8 and 10 mL of ethyl acetate, then, centrifuged at 15,000 rpm for 15 min at 4 °C. The upper fraction was used for total antioxidant activity due to lipophilic compounds (L-TAA) and the lower for total antioxidant activity due to hydrophilic compounds (H-TAA). In both cases, TAA was determined using the enzymatic system composed of the chromophore 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), the horse radish peroxidase enzyme (HRP) and its oxidant substrate (hydrogen peroxide,  $\text{H}_2\text{O}_2$ ), in which  $\text{ABTS}^{\bullet+}$  radicals are generated and monitored at 730 nm. The reaction mixture contained 5 mM ABTS, 5  $\mu\text{M}$   $\text{H}_2\text{O}_2$  and 5  $\mu\text{M}$  HRP in 50 mM glycine buffer (pH = 4.5) in a total volume of 1 mL. The decrease in absorbance after adding the extract was proportional to TAA of the sample. A calibration curve was performed with Trolox [(R)-(+)-6-hydroxy-2,5,7,8-tetramethyl-croman-2-carboxylic acid] (0–20 nmol) from Sigma (Madrid, Spain), and results are expressed as mg of Trolox equivalent  $100 \text{ g}^{-1} \text{ fw}$ .

### 2.5. Fermented Beverages Analysis

#### 2.5.1. Physico-Chemical and Microbiology Analysis

The CIEL  $a^*b^*$  color space of fermented milk was studied, and the following color coordinates were evaluated: lightness ( $L^*$ ), redness ( $a^*$ , green-red coordinate), and yellowness ( $b^*$ , blue-yellow coordinate). Color determinations were made at  $12 \pm 2$  °C by means of a Minolta CRC-200 (Minolta Camera Co. Osaka, Japan) spectrophotometer, with a liquid accessory CR-A70 (Minolta Camera Co. Osaka, Japan), with illuminated D65 and an observer of 10 °C. The equipment was daily calibrated with the white plate provided by Minolta. pH was determined by a pH-meter Crison GLP 21 (Crison Instruments, S.A., Barcelona, Spain). MRS agar was used for lactobacilli counts: *L. paracasei* at 30 °C for 72 h under aerobic conditions, *L. casei* at 37 °C for 72 h under aerobic conditions and *L. helveticuss* at 37 °C for 72 h in a candle jar, always following directions of CECT. Three replicates were run for pH and microbial counts, and nine for color.

#### 2.5.2. Total Phenolics, Anthocyanin Determination and Total Antioxidant Activity

Total phenolics and anthocyanins were determined following the same procedures described for plant material. Total antioxidant activity was quantified as described for the plant material but using 5 g fermented milk instead of 2 g.

### 2.6. Statistical Analysis

Statistical analysis and comparison among means were carried out using the statistical package SPSS 24.0 (IBM SPSS Statist cs, Chicago, IL, USA). Multivariate General Linear Model Procedure was used to evaluate factors. Tukey's test was used for means comparison (95% confidence level).

## 3. Results and Discussion

In the present sections results will be presented focusing on the properties of the beverages, so, color, pH and microbial counts of the beverages will be presented first, followed by antioxidant properties and anthocyanin content in fruits and beverages to discuss the effects of fermentation and storage on the main parameters of the study.

### 3.1. Physico-Chemical Parameters and Microbiology of the Fermented Beverages

Table 1 shows the results of the color values and Table 2 reports the results of the multifactor analysis of variance for the color of fermented milks [starter: *L. helveticuss* CECT 541, *L. casei* CETC 475 and *L. paracasei* CECT 277; ripening stage of sweet cherry: commercial ripening (stage 8) and over ripening (stage 12)].

The results of the statistical study showed that  $L^*$  parameter was affected by both the LAB and the ripening stage of the cherry. However,  $L^*$  values were only slightly modified (Tables 1 and 2). According to García-Pérez et al. [17], lightness in fermented milk decrease as pH decreases, due to gelation, in the present study the addition over ripened fruit also decreased lightness.

**Table 1.** Color parameters color (average  $\pm$  standard deviation) of fermented milks with sweet cherry puree at different stages of ripening (8 and 12) before fermentation (0) and at days 1 and 10 of refrigerated storage.

Color	Cherry Ripening Stage		Time (Days)		
			0	1	10
<i>L. helveticuss</i> CECT 541	8	$L^*$	33.76 $\pm$ 0.06	33.39 $\pm$ 0.06	32.52 $\pm$ 0.51
		$a^*$	0.16 $\pm$ 0.02	0.82 $\pm$ 0.05	1.23 $\pm$ 0.12
		$b^*$	3.94 $\pm$ 0.02	6.17 $\pm$ 0.09	6.60 $\pm$ 0.22
	12	$L^*$	28.24 $\pm$ 0.04	27.14 $\pm$ 0.25	27.85 $\pm$ 0.35
		$a^*$	1.96 $\pm$ 0.05	4.07 $\pm$ 0.15	3.88 $\pm$ 0.06
		$b^*$	3.11 $\pm$ 0.03	5.46 $\pm$ 0.10	5.99 $\pm$ 0.12
<i>L. casei</i> CECT 475	8	$L^*$	34.31 $\pm$ 0.04	34.95 $\pm$ 0.33	33.86 $\pm$ 0.07
		$a^*$	0.02 $\pm$ 0.02	0.61 $\pm$ 0.05	0.67 $\pm$ 0.06
		$b^*$	4.08 $\pm$ 0.03	6.83 $\pm$ 0.05	6.75 $\pm$ 0.06
	12	$L^*$	28.30 $\pm$ 0.04	29.74 $\pm$ 0.28	27.34 $\pm$ 0.07
		$a^*$	1.96 $\pm$ 0.01	3.55 $\pm$ 0.04	3.60 $\pm$ 0.08
		$b^*$	2.83 $\pm$ 0.04	6.14 $\pm$ 0.13	5.37 $\pm$ 0.07
<i>L. paracasei</i> CECT 277	8	$L^*$	33.94 $\pm$ 0.25	27.85 $\pm$ 0.35	33.17 $\pm$ 1.01
		$a^*$	0.05 $\pm$ 0.05	0.63 $\pm$ 0.09	0.89 $\pm$ 0.13
		$b^*$	4.22 $\pm$ 0.05	6.85 $\pm$ 0.14	6.67 $\pm$ 0.13
	12	$L^*$	26.89 $\pm$ 0.47	28.04 $\pm$ 0.55	28.64 $\pm$ 0.16
		$a^*$	1.84 $\pm$ 0.13	3.81 $\pm$ 0.15	3.55 $\pm$ 0.06
		$b^*$	2.84 $\pm$ 0.02	5.23 $\pm$ 0.10	5.80 $\pm$ 0.09

$L^*$  (lightness),  $a^*$  (green-red coordinate) and  $b^*$  (blue-yellow coordinate).

**Table 2.** Homogeneous groups as determined by Tukey's multiple range test ( $p < 0.05$ ) for color ( $L^*$ ,  $a^*$  and  $b^*$  coordinates) determined in fermented milks with addition of sweet cherry, independent factors: starter culture (*L. helveticuss*, *L. casei*, *L. paracasei*), cherry ripening stage (8: with addition of sweet cherry in a ripe state 8; 12: with addition of sweet cherry in a ripe state 12) and time (0 pre-fermentation, and 1, and 10 days of refrigerated storage).

	Starter			Cherry Ripening Stage		Time		
	<i>L. helveticuss</i>	<i>L. casei</i>	<i>L. paracasei</i>	8	12	0	1	10
$L^*$	a	b	a	b	a	a	b	a
$a^*$	b	a	b	a	b	a	b	b
$b^*$	a	c	b	b	a	a	b	c

Different letters in the same row are indicative of significant differences ( $p < 0.05$ ) among mean values, with "a" being the lowest value and increasing according to alphabetical order.  $L^*$  (lightness),  $a^*$  (green-red coordinate) and  $b^*$  (blue-yellow coordinate).

With respect to the parameter  $a^*$ , the fermentation with *L. helveticuss*, *L. casei* and *L. paracasei*, followed a strong tendency, independently of the ripening stage of cherries. The use of cherries at

ripening stage 12 yielded higher  $a^*$  values (Table 1). It can be appreciated that this parameter increased during storage time for all fermented milks, which indicates browning of sweet cherry puree in these 10 days. García-Pérez et al. [17], in yogurts with orange fiber, observed that the syneresis observed in the fermented milks produced a decrease of  $a^*$  values due to the fact that the milk serum released by the gel contains riboflavin, which has a very important green component; however, the addition of cherry fiber was very colored and, therefore, high values of  $a^*$  were obtained. Trigueros et al. [18], also showed an increase in the level of redness during refrigerated storage in yogurts with added pomegranate, agreeing with the results observed in the current experiment.

Regarding yellowness, the parameter  $b^*$  (yellowness component if  $b^* > 0$ ) followed a trend similar to that previously reported for the coordinate  $a^*$  (Table 1). In all fermented milks (different lactic bacteria and ripening stages), parameter  $b^*$  increased from day 0 to day 10. This, together with the increase of the parameter  $a^*$  indicated a shift of the fermented milks towards either brown tones for cherries at stage 8 or to violet tones for cherries at stage 12. This increase in the parameter  $b^*$  may be due to the release of pigments by the sweet cherry, as happened to García-Pérez et al. [17], with the orange carotenes. On the other hand, according to Serrano et al. [5], regarding the external color parameters of the cherry fruit, lightness ( $L^*$ ) and  $b^*$  values were highest at early stages of development, with a linear decrease until stage 10, and, then, being constant until the end of ripening. The color of the anthocyanins depends on the chemical substituents that they contain and their position in the flavial group; that is, if the hydroxyl of the phenolic ring is increased, the blue color intensifies, while the introduction of methoxyl causes the formation of the red color [7]. This observation is linked with the pH changes of the milks, making obvious that the color of fermented milks depends on their pH. Therefore, the decrease in pH, caused by the fermentation of microorganisms could have caused an increase of the hydroxylation of the phenolic ring, and, therefore, a change of pigments towards blueish tones.

Table 3 reports the results of pH parameters and microbiological counts for each type of fermented milk [starter: *L. helveticuss* CECT 541, *L. casei* CETC 475 and *L. paracasei* CECT 277; ripening stage of sweet cherry: commercial ripening (stage 8) and over ripening (stage 12)], and Table 4 shows the parameters studied with significant differences ( $p < 0.05$ ), to which the TUKEY test is applied to compare the means.

The results of the statistical analysis pointed out significant differences ( $p < 0.05$ ) for pH due to all variables under analysis: starter, type of sweet cherry puree, and time. *L. helveticuss* fermentation yielded the fermented milks with the lowest pH, followed by *L. paracasei*, with the highest pH corresponding to *L. casei*. In general, the presence of sweet cherry led to lower pH values as compared to the control samples, indicating that the presence of sweet cherry acidified the milks, enhanced the acidification rate and in the case of *L. casei* also the microbial growth. Acidification continued during refrigerated storage, as expected.

Cherry pure microbial load was under  $2 \log \text{cfu g}^{-1}$  for mesophilic bacteria and molds and yeasts. Such low counts together with the inoculation of LAB (Table 3, counts day 0) and incubation conditions favoring milk fermentation may enable mechanisms of lactic antagonism to avoid the significant growth of competitors. Counts detected on MRS may be mainly be attributable to the inoculated LAB cultures. Microbial counts were also affected by all variables, although quite similar among them, and the highest counts were obtained for *L. paracasei* and *L. casei*. Interestingly, *L. helveticuss*, despite leading to the lowest pH, was not the one with the largest microbial populations; this was probably due to a greater capacity to metabolize both fruit and milk sugars. The presence of sweet cherry significantly increased the microbial populations, especially at the over ripening stage, which is probably due to the largest content in hexoses. Microbial counts were also affected by time: at day 1 the highest counts were obtained, and, then, they decreased slightly, but the decrease was significant at day 10 (Table 3). Over-ripe cherries have higher sugar and acid content than cherries at commercial stage, being glucose and fructose with 6.3 and 7.7%, respectively, the main sugars, and malic and ascorbic acids with 1.2

and 0.82% respectively, the main organic acids [5]. This high content of glucose and fructose, which can be metabolized by all tested bacteria [19], may have favored microbial growth.

**Table 3.** pH and microbial counts parameters (average and standard deviation) of fermented milks with sweet cherry puree at different stages of maturation (8 and 12) before fermentation (0) and at days 1 and 10 of refrigerated storage.

Starters	Cherry Ripening Stage	Time (Days)		
		0	1	10
pH				
<i>L. helveticuss</i> CECT 541	Control	6.64 ± 0.08	3.52 ± 0.02	3.75 ± 0.05
	8	6.01 ± 0.01	3.59 ± 0.05	3.72 ± 0.07
	12	6.16 ± 0.01	3.55 ± 0.02	3.71 ± 0.08
<i>L. casei</i> CECT 475	Control	6.66 ± 0.02	4.98 ± 0.21	4.54 ± 0.03
	8	6.05 ± 0.03	4.15 ± 0.01	4.03 ± 0.02
	12	6.20 ± 0.01	4.14 ± 0.04	3.96 ± 0.05
<i>L. paracasei</i> CECT 277	Control	6.68 ± 0.01	5.27 ± 0.12	4.24 ± 0.06
	8	6.05 ± 0.02	3.91 ± 0.04	3.92 ± 0.04
	12	6.15 ± 0.01	3.90 ± 0.03	3.93 ± 0.01
Microbial counts (log cfu mL <sup>-1</sup> )				
<i>L. helveticuss</i> CECT 541	Control	6.92 ± 0.01	9.14 ± 0.09	8.60 ± 0.03
	8	6.92 ± 0.01	9.28 ± 0.02	8.61 ± 0.05
	12	6.92 ± 0.01	9.59 ± 0.07	8.91 ± 0.20
<i>L. casei</i> CECT 475	Control	6.83 ± 0.04	9.14 ± 0.05	8.67 ± 0.08
	8	6.83 ± 0.04	9.66 ± 0.54	9.22 ± 0.04
	12	6.83 ± 0.04	9.48 ± 0.12	9.28 ± 0.10
<i>L. paracasei</i> CECT 277	Control	6.61 ± 0.03	9.35 ± 0.04	9.42 ± 0.17
	8	6.61 ± 0.03	9.48 ± 0.02	9.29 ± 0.08
	12	6.61 ± 0.03	9.77 ± 0.31	9.31 ± 0.08

**Table 4.** Homogeneous groups as determined by Tukey's multiple range test ( $p < 0.05$ ) for pH and microbial counts determined in fermented milks with addition of sweet cherry, independent factors: starter culture (*L. helveticuss*, *L. casei*, *L. paracasei*), cherry ripening stage (control: without addition of sweet cherry; 8: with addition of sweet cherry in a ripe stage 8; 12: with addition of sweet cherry in a ripe stage 12) and time (0 pre-fermentation, and 1, and 10 days of refrigerated storage).

	Starters			Cherry Ripening Stage			Time		
	<i>L. helveticuss</i>	<i>L. casei</i>	<i>L. paracasei</i>	Control	8	12	0	1	10
pH	a	c	b	c	a	b	c	b	a
Microbial counts	a	ab	b	a	ab	b	a	c	b

Different letters in the same row are indicative of significant differences ( $p < 0.05$ ) among mean values, with "a" being the lowest value and increasing according to alphabetical order.

pH decreased in milk fermented by *L. helveticuss* following the same trend, regardless of the addition or not of sweet cherry puree and its ripening stage (Tables 3 and 4). Therefore, it was clear that both the control milk and the fermented milks with cherry puree at ripening stage 8 and 12, decreased on the first day to a pH close to 3.5, and in later days increased slightly until pH 3.7. *L. casei* and *L. paracasei* fermented milks acidification was favored by the addition of cherry puree. As shown in Tables 3 and 4, fermented milks with *L. casei* and *L. paracasei*, with cherry at both ripening stages 8 and 12, presented similar changes of pH. In studies with citrus fiber, no significant differences were detected for the pH due to storage time or the presence of fiber [8,20].

*L. helveticuss* had the highest acidification ability, regardless the presence of cherry, reaching a pH close to 3.5. The microorganisms favored by the addition of sweet cherry puree (at both ripening stages) were *L. casei* and *L. paracasei*. Conversely, Trigueros et al. [18], observed that yogurts rich in pomegranate had a higher pH compared to control yogurts, probably related to the profile of phenolic compounds in pomegranate which slightly reduced counts of lactic acid bacteria. Counts of *streptococci* decreased (1 log unit) due to pomegranate addition, whereas *lactobacilli* counts were not affected by the presence of pomegranate juice. Still, total LAB counts were over  $10^8$  cfu g<sup>-1</sup> in such yogurts. In addition, Cano-Lamadrid et al. [8] also reported that the pH of fermented milks decreased with the addition of pomegranate juice and such addition resulted in an enhancement of LAB growth depending on the strain.

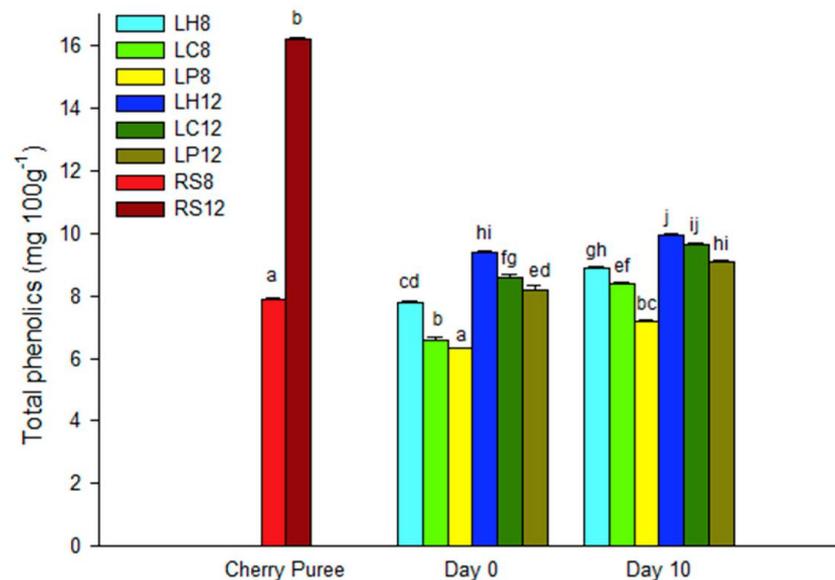
As shown in Tables 3 and 4, the addition of sweet cherry puree and its ripening stage influenced the growth of the three lactic acid bacteria. During the first days of fermentation, *L. helveticuss* and *L. paracasei*, showed a higher initial growth for fermented milk with cherries at over ripening stage, although the counts of *L. helveticuss* were significantly lower than those of *L. paracasei*. The growth of both microorganisms was lower in the control and the milk with puree at stage 8, being the counts of the latter superior to the control. However, *L. casei* showed a higher growth in milk with puree at stage 8 and lower counts for control milk and milk with puree at stage 12. Sendra et al. [20] showed that the addition of citrus fiber contributes to an increase in the survival of *L. casei* above  $8 \log$  CFU mL<sup>-1</sup>, revealing that fermented milks enriched with citrus fiber are good vehicles for incorporating probiotic bacteria. This microorganism had experienced the same behavior in the presence of sweet cherry puree in stage 8. Lactic bacteria do not behave in the same way in the presence of all fruits; for example, Trigueros et al. [18] observed that yogurt rich in pomegranate had lower counts of lactic acid bacteria compared to control yogurt.

As the fermentation process advanced, the counts of *L. helveticuss*, *L. casei* and *L. paracasei*, independently of the addition of puree and the ripening stage, reached approximately similar values. *L. casei* was the most sensitive starter to the addition of puree, and *L. helveticuss* the one that resulted in the lowest counts. The three lactic acid bacteria used here can metabolizing fructose [21]. Thus, it would be expected that all of them would have grown better in the presence of over ripe cherry (ripening stage 12) because fruits have more fructose [5] and more total sugars 14.3% compared to 9% at stage 8; however, *L. casei* grew better in the presence of sweet cherry in a ripening stage 8. In both types of sweet cherries, the sugar content was high and did not suppose a limiting factor for bacterial growth. TPC of cherries in a ripening stage of 12 was higher than that at 8, these phenolic compounds may have also had a different impact on *L. casei* as compared to the other evaluated LAB, as the resistance of bacteria to these compounds is variable from strain to strain. In general, the sweet cherry at a ripening stage 12 have higher contents of organic acids and sugars than at commercial ripening (stage 8), but above all the contents of glucose, fructose and ascorbic acid are high [5]. In addition, it has been demonstrated that the incorporation of fruit to dairy products maintains or increases the growth and survival of probiotics in dairy products [8,20,22–24].

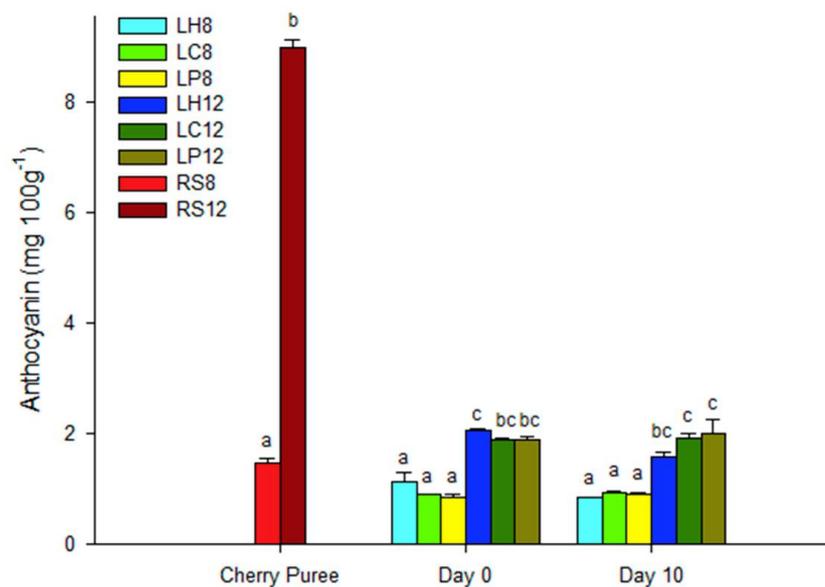
### 3.2. Total Phenolics, Anthocyanin Determination and Total Antioxidant Activity of the Fermented Beverages

After the addition of 15% of sweet cherry (stage 8 and 12) to the milk, the total phenolic content (TPC) was  $7.88 \pm 0.07$  and  $16.2 \pm 0.05$  mg per 100 g, respectively (cherry puree: ripening stage 8, RS8 and ripening stage 12, RS12); being these TPC values in agreement with other studies in which this content increased with the ripening of fruit regardless of the cultivar [2]. Figure 1 shows that the process of fermentation and storage (days 0 versus 10) influenced the TPC with a slight increase over the storage time, with the highest values being found in samples with sweet cherry puree at stage 12. For both ripening stages, milks with *L. helveticuss* had the highest values of TPC the two sampling days, while *L. paracasei* had the lowest ones. On the other hand, TPC in FM at day 0 were higher than expected by the dilution factor of cherry pure in milk (15%), so other factors may had interfered in the colorimetric determination of TPC. Phenolic content in fermented milk is affected by pH, initial content of the fruit

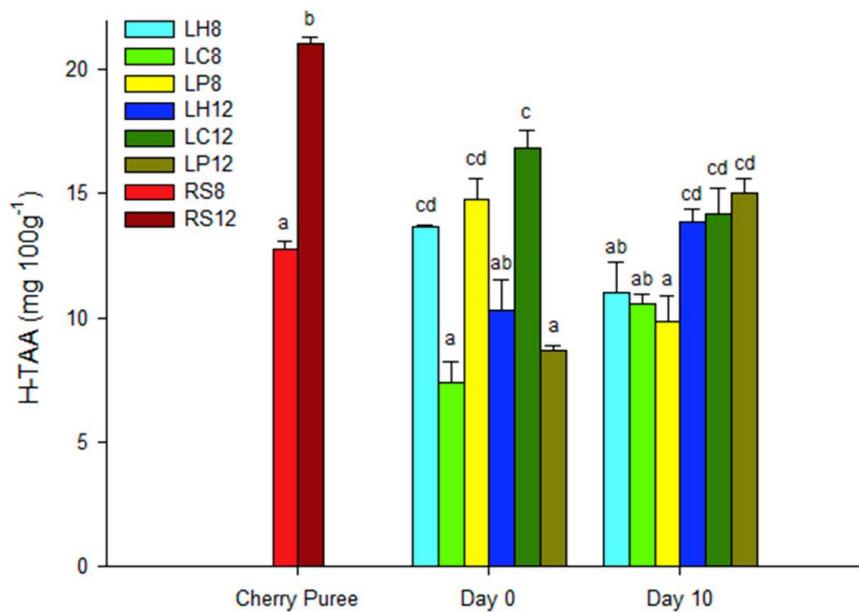
and bacterial strains among others [9]. Besides, at day 0, since there are no differences in pH, counts, or storage time, the differences presents in the TPC could be due to interactions of phenols with milk proteins [9]. It is the same case for anthocyanin determination and total antioxidant activity (Figures 2–4).



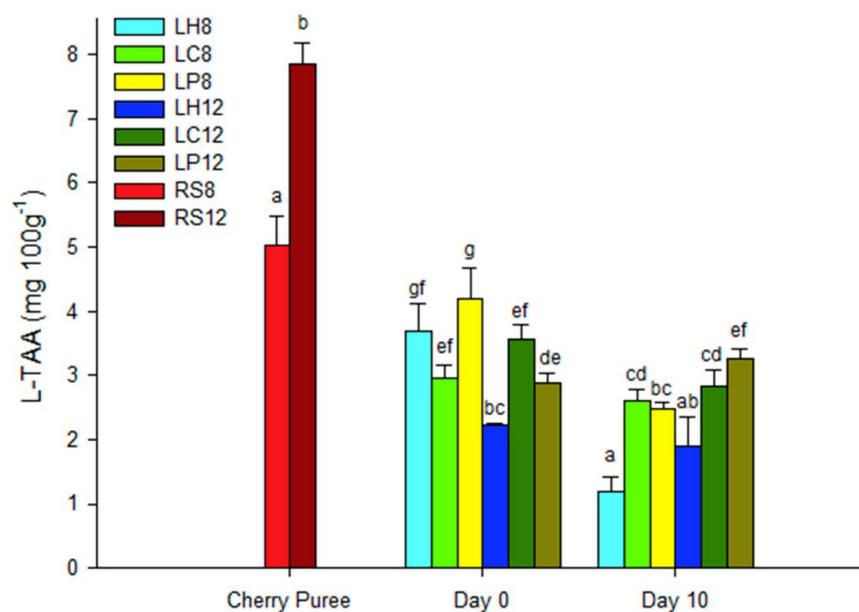
**Figure 1.** Concentration of total phenols ( $\text{mg } 100 \text{ g}^{-1}$ ) of sweet cherry puree before incorporation [RS8: ripening stage 8; RS12: ripening stage 12] and in fermented milk by LH: *Lactobacillus helveticus*, LC: *Lactobacillus casei*, and LP: *Lactobacillus paracasei* with sweet cherry puree at ripening stage 8: commercial ripening; 12: over ripening. Different letters indicative of significant differences ( $p < 0.05$ ) among mean values, with “a” being the lowest value and increasing according to alphabetical order. Error bars represent the standard deviations.



**Figure 2.** Concentration of total anthocyanins ( $\text{mg } 100 \text{ g}^{-1}$ ) of sweet cherry puree before incorporation [RS8: ripening stage 8; RS12: ripening stage 12] and in fermented milk by LH: *Lactobacillus helveticus*, LC: *Lactobacillus casei*, and LP: *Lactobacillus paracasei* with sweet cherry puree at ripening stage 8: commercial ripening; 12: over ripening. Different letters indicative of significant differences ( $p < 0.05$ ) among mean values, with “a” being the lowest value and increasing according to alphabetical order. Error bars represent the standard deviations.



**Figure 3.** Total antioxidant activity due to hydrophilic compounds (H-TAA) ( $\text{mg } 100 \text{ g}^{-1}$ ) of sweet cherry puree before incorporation [RS8: ripening stage 8; RS12: ripening stage 12] and in fermented milk by LH: *Lactobacillus helveticus*, LC: *Lactobacillus casei*, and LP: *Lactobacillus paracasei* with sweet cherry puree at ripening stage 8: commercial ripening; 12: over ripening. Different letters indicative of significant differences ( $p < 0.05$ ) among mean values, with “a” being the lowest value and increasing according to alphabetical order. Error bars represent the standard deviations.



**Figure 4.** Total antioxidant activity due to lipophilic compounds (L-TAA) ( $\text{mg } 100 \text{ g}^{-1}$ ) of sweet cherry puree before incorporation [RS8: ripening stage 8; RS12: ripening stage 12] and in fermented milk by LH: *Lactobacillus helveticus*, LC: *Lactobacillus casei*, and LP: *Lactobacillus paracasei* with sweet cherry puree at ripening stage 8: commercial ripening; 12: over ripening. Different letters indicative of significant differences ( $p < 0.05$ ) among mean values, with “a” being the lowest value and increasing according to alphabetical order. Error bars represent the standard deviations.

Fermented milks with sweet cherry puree at stage 12 had significantly higher contents of anthocyanins than samples with puree fruit at stage 8 (Figure 2), because of the anthocyanin content at each ripening stage of the fruit. Similar to phenols, these results are consistent with the observation of Díaz-Mula et al. [6] and Serrano et al. [2], in which the anthocyanins and TPC contents increased exponentially with fruit ripening Serrano et al. [5] from stage 8 to stage 14. The anthocyanins remain approximately stable over time in the milk fermented by *L. casei* and *L. paracasei*, independently of the ripening stage of the added cherries and experienced a notable decrease in the case of *L. helveticuss*. The structure of the anthocyanins influences their stability during the fermentation process, with monoglycosides being less stable than diglycosides. The anthocyanin cyanidin-3-O-glucoside, predominant in cherries [5], is the most affected in the fermentation process [9]; thus, a decrease of anthocyanins is expected to be found after the fermentation of milks. However, Trigueros et al. [9] revealed that most anthocyanins remain bound to milk proteins because the flavonoid monoglycosides show strong binding affinities with these proteins; which may have somehow protected anthocyanins during storage.

With respect to total antioxidant activity in both fractions, hydrophilic and lipophilic (H-TAA, L-TAA) increases throughout the maturation process of sweet cherries [6]. In the case of fermented milks, H-TAA was higher than L-TAA, because hydrophilic compounds (e.g., phenols and anthocyanins) are present in higher amount than lipophilic compounds [6,16]. In general, the addition of sweet cherry puree to fermented milks resulted in a dilution of antioxidant compounds and so in a decrease in H-TAA (Figure 3). H-TAA was not modified during refrigerated storage.

The L-TAA showed a generalized decrease as storage advanced (Figure 4), which agreed with the decrease in the antioxidant activity of pomegranate polyphenols reported by Trigueros et al. [9]. These authors explained the decrease in the antioxidant activity by increased interactions among proteins-polyphenols during storage time. However, this effect was not observed in milk fermented by *L. paracasei* with over-ripe cherries puree (stage 12).

#### 4. Conclusions

The addition of cherry puree stimulated acidification in milks fermented by *Lactobacillus casei* and *Lactobacillus paracasei*. Acidification by *Lactobacillus helveticus* was not affected by the presence of cherry, although it is the lactic acid bacteria demonstrating the greatest acidifying activity. The strains with best viability during 10 days of storage were *Lactobacillus casei* and *Lactobacillus paracasei*. However, all tested LAB yield counts values over  $6 \log \text{cfu g}^{-1}$  and all bacteria demonstrated great acidifying activity, were, therefore, suitable for preparation of commercial fermented milks.

Fermented milks with cherry puree experienced color changes with storage time leading to brown color for stage 8 cherries and to violet color for stage 12 cherries. Fermented milks with cherry puree at stage 12 of ripening better preserved phenolic compounds and total anthocyanins after 10 days of storage. *L. helveticuss* better retained the content of phenols, and *L. casei* and *L. paracasei* that of anthocyanins after 10 days of refrigerated storage.

Therefore, for the development of fermented milks with cherry puree it would be interesting to use *Lactobacillus helveticus* because it is a strain of rapid acidification, with adequate counts, high intensity of red color ( $a^*$  coordinate), in addition the preservation of total phenols, and the loss of anthocyanins and water-soluble antioxidant activity was minimal. It would also be interesting to use the cherry puree at stage 12, due to its higher content of phenols, anthocyanins and greatest total antioxidant activity.

When fruits are to be used in fermented milks, it is of great relevance to determine the effect of LAB culture and stage of maturity of the fruit on the preservation of bioactive compounds and antioxidant activities. In this way, the developed product may provide best color, nutrition and health related properties. It is well recognized that fermentation has an enormous potential to improve the bioaccessibility and the bioactivity of polyphenol compounds. For instance, further studies are needed for the characterization of the phenolic derivatives generated during the fermentation process.

**Author Contributions:** P.S.-B. run laboratory work and wrote the first draft of the manuscript; author A.M.-E. worked on the field and laboratory analysis of vegetable material; author P.J.Z. advised on the use and analysis of vegetable material and supervised and analyzed vegetable data; author Á.A.C.-B. revised the manuscript and data; author E.S. designed the study of the fermented milks and run statistical analysis of dairy data. All authors revised the finished manuscript and corrections.

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## References

1. McCune, L.M.; Kubota, C.; Stendell-Hollis, N.R.; Thomson, C.A. Cherries and health: A review. *Crit. Rev. Food Sci. Nutr.* **2011**, *51*, 1–12. [CrossRef] [PubMed]
2. Serrano, M.; Diaz-Mula, H.M.; Zapata, P.J.; Castillo, S.; Guillén, F.; Martínez-Romero, D.; Valverde, J.M.; Valero, D. Maturity stage at harvest determines the fruit quality and antioxidant potential after storage of sweet cherry cultivars. *J. Agric. Food Chem.* **2009**, *57*, 3240–3246. [CrossRef] [PubMed]
3. Stintzing, F.C.; Carle, R. Functional properties of anthocyanins and betalains in plants, food, and in human nutrition. *Trends Food Sci. Technol.* **2004**, *15*, 19–38. [CrossRef]
4. Goulao, L.F.; Oliveira, C.M. Cell wall modifications during fruit ripening: When a fruit is not the fruit. *Trends Food Sci. Technol.* **2008**, *19*, 4–25. [CrossRef]
5. Serrano, M.; Guillén, F.; Martínez-Romero, D.; Castillo, S.; Valero, D. Chemical constituents and antioxidant activity of sweet cherry at different ripening stages. *J. Agric. Food Chem.* **2005**, *53*, 2741–2745. [CrossRef] [PubMed]
6. Díaz-Mula, H.M.; Castillo, S.; Martínez-Romero, D.; Valero, D.; Zapata, P.J.; Guillén, F.; Serrano, M. Sensory, nutritive and functional properties of sweet cherry as affected by cultivar and ripening stage. *Food Sci. Technol. Int.* **2009**, *15*, 535–543. [CrossRef]
7. Aguilera, M.; Reza, M.C.; Gerardo, R.; Madinaveitia, C.; Meza, J.A. Functional properties of anthocyanins. *Ciencias biológicas y de la salud de la Universidad de Sonora* **2011**, *13*, 16–22.
8. Cano-Lamadrid, M.; Trigueros, L.; Wojdyło, A.; Carbonell-Barrachina, Á.A.; Sendra, E. Anthocyanins decay in pomegranate enriched fermented milks as a function of bacterial strain and processing conditions. *LWT Food Sci. Technol.* **2017**, *80*, 193–199. [CrossRef]
9. Trigueros, L.; Wojdyło, A.; Sendra, E. Antioxidant activity and protein-polyphenol interactions in a pomegranate (*Punica granatum* L.) yogurt. *J. Agric. Food Chem.* **2014**, *62*, 6417–6425. [CrossRef] [PubMed]
10. Batt, C.A. *Lactobacillus*, introduction. In *Encyclopedia of Food Microbiology*; Robinson, R.K., Ed.; Elsevier: Oxford, UK, 1999; pp. 1134–1136.
11. Sendra, E.; Sayas-Barberá, E.; Fernández-López, J.; Pérez-Álvarez, J. Effect of food composition on probiotic bacteria viability. *Bioact. Foods Health Promot.* **2016**, *17*, 257–269.
12. Gobbetti, M. *Lactobacillus*, *Lactobacillus casei*. In *Encyclopedia of Food Microbiology*; Robinson, R.K., Ed.; Elsevier: Oxford, UK, 1999; pp. 1157–1164.
13. MAPAMA. Informe del Consumo de Alimentación en España 2016. Available online: [http://www.mapama.gob.es/images/es/informe\\_del\\_consumo\\_de\\_alimentos\\_en\\_espana\\_2016\\_web\\_tcm30-419484.pdf](http://www.mapama.gob.es/images/es/informe_del_consumo_de_alimentos_en_espana_2016_web_tcm30-419484.pdf) (accessed on 25 May 2018).
14. Augustin, M.; Clarke, P.T.; Craven, H. Powdered milk: Characteristics of milk powders. In *Encyclopaedia of Food Science and Nutrition*, 2nd ed.; Caballero, B., Ed.; Elsevier Science: London, UK, 2003; pp. 4703–4711.
15. Tomás-Barberán, F.A.; Gil, M.I.; Cremin, P.; Waterhouse, A.L.; Hess-Pierce, B.; Kader, A.A. Hplc-dad-esims analysis of phenolic compounds in nectarines, peaches, and plums. *J. Agric. Food Chem.* **2001**, *49*, 4748–4760. [CrossRef] [PubMed]
16. Martínez-Esplá, A.; Zapata, P.J.; Valero, D.; García-Viguera, C.; Castillo, S.; Serrano, M. Preharvest application of oxalic acid increased fruit size, bioactive compounds, and antioxidant capacity in sweet cherry cultivars (*Prunus avium* L.). *J. Agric. Food Chem.* **2014**, *62*, 3432–3437. [CrossRef] [PubMed]

17. García-Pérez, F.J.; Lario, Y.; Fernández-López, J.; Sayas, E.; Pérez-Alvarez, J.A.; Sendra, E. Effect of orange fiber addition on yogurt color during fermentation and cold storage. *Color Res. Appl.* **2005**, *30*, 457–463. [[CrossRef](#)]
18. Trigueros, L.; Viuda-Martos, M.; Perez-Alvarez, J.A.; Sendra, E. Low fat set yoghurt rich in pomegranate juice: A new antioxidant dairy product. *Milchwissenschaft* **2012**, *67*, 177–180.
19. Limsowtin, G.K.Y.; Broome, M.C.; Powell, I. *Lactic Acid Bacteria, Taxonomy*; Encyclopedia of Dairy Sciences, Academic Press: London, UK, 2002; pp. 1470–1478.
20. Sendra, E.; Fayos, P.; Lario, Y.; Fernández-López, J.; Sayas-Barberá, E.; Pérez-Alvarez, J. Incorporation of citrus fibers in fermented milk containing probiotic bacteria. *Food Microbiol.* **2008**, *25*, 13–21. [[CrossRef](#)] [[PubMed](#)]
21. Sharpe, M.E.; Wheeler, D.M. *Lactobacillus helveticus*. *J. Gen. Microbiol.* **1957**, *16*, 676–679. [[CrossRef](#)] [[PubMed](#)]
22. Bueno, L.; Silva, T.; Perina, N.; Bogsan, C.; Oliveira, M. Addition of strawberry, raspberry and “pitanga” pulps improves the physical properties of symbiotic yoghurts. *Chem. Eng.* **2014**, *38*, 499–504.
23. Espirito-Santo, A.P.; Cartolano, N.S.; Silva, T.F.; Soares, F.; Gioielli, L.; Peregó, P.; Converti, A.; Oliveira, M. Fibers from fruit by-products enhance probiotic viability and fatty acid profile and increase CLA content in yoghurts. *Int. J. Food Microbiol.* **2012**, *154*, 135–144. [[CrossRef](#)] [[PubMed](#)]
24. Rotar, A.; Vodnar, D.; Bunghez, F.; Catunescu, G.; Pop, C.; Jimborean, M.; Semeniuc, C. Effect of goji berries and honey on lactic acid bacteria viability and shelf life stability of yoghurt. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca* **2015**, *43*. [[CrossRef](#)]



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