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Aguamiel Enhance Proteolytic Activity and Survival of *Lactiplantibacillus pentosus* ABHEAU-05 during Refrigerated Storage of a Fermented Milk

Elizabeth Contreras-López [1], Nancy Lizeth Félix-Reyes [1], Luis Guillermo González-Olivares [1,*], Judith Jaimez-Ordaz [1], Araceli Castañeda-Ovando [1], Javier Añorve-Morga [1], Blanca Azalia López-Hernández [2], Nayeli Vélez-Rivera [2] and Juan Ramírez-Godínez [2,*]

[1] Área Académica de Química, Instituto de Ciencias Básicas e Ingeniería, Universidad Autónoma del Estado de Hidalgo, Mineral de la Reforma 42184, Hidalgo, Mexico; elizac@uaeh.edu.mx (E.C.-L.); nancyrs.dl@gmail.com (N.L.F.-R.); jjaimez@uaeh.edu.mx (J.J.-O.); ovandoa@uaeh.edu.mx (A.C.-O.); anorvej@uaeh.edu.mx (J.A.-M.)

[2] Área Académica de Gastronomía, Instituto de Ciencias Económico Administrativas, Universidad Autónoma del Estado de Hidalgo, Circuito La Concepción Km 2.5 Col. San Juan Tilcuautla, San Agustín Tlaxiaca 42160, Hidalgo, Mexico; blanca_lopez@uaeh.edu.mx (B.A.L.-H.); nayeli_velez@uaeh.edu.mx (N.V.-R.)

* Correspondence: lgonzales@uaeh.edu.mx (L.G.G.-O.); juan_ramirez@uaeh.edu.mx (J.R.-G.); Tel.: +52-771-7172000 (ext. 2692) (J.R.-G.)

Abstract: Different carbon sources, such as prebiotics, have promoted probiotics' survival during refrigerated fermented milk storage. These compounds stimulate both the metabolic response and the resistance of probiotics to adverse conditions, such as low temperatures. That is why the objective of this study was to evaluate the kinetic parameters of growth, the proteolytic profile, and the survival of *Lactiplantibacillus pentosus* ABHEAU-05 in fermented milk added with aguamiel as a prebiotic source during refrigerated storage. Inulin was used for control experiments. A 12% *w/v* powdered skimmed milk solution was inoculated with *L. pentosus* ABHEAU-05 (10^6 CFU/mL). It was fermented at 37 °C until a pH of 4.5, and the kinetic parameters were calculated. Analysis of survival and proteolytic profile during refrigeration storage (4 °C for 21 days) was carried out. The survival of the microorganism was determined by viable count on MRS agar, the production of free amino groups by the TNBS method, and the accumulation of low molecular weight peptides by polyacrylamide gel electrophoresis (SDS-PAGE). The pH of 4.5 was reached 26 h before the control. The maximum concentration of viable cells was 10^8 CFU/rmL at the fermentation's end and maintained throughout the refrigerated storage. With the analysis of the proteolytic profile, high metabolic activity was demonstrated during fermentation and refrigerated storage in milk with aguamiel. The accumulation of low molecular weight peptides and the generation of free amino groups were higher than the control results. It was verified that aguamiel is a carbon source with the potential for developing and maintaining the probiotic *L. pentosus* ABHEAU-05 in fermented milk.

Keywords: aguamiel; probiotic; prebiotic; fermented milk; peptides

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1. Introduction

Currently, it is sought that foods satisfy consumers' needs while offering health benefits. For this reason, the so-called functional foods have had a significant impact in recent years since, in addition to providing nutrients, they beneficially affect one or more human functions [1]. The nutraceutical effect has been demonstrated through different studies on the modulation of the physiological, endocrine, circulatory, and digestive systems [2,3].

One of the main functional foods that have had relevance due to its high acceptability by the consumer and a wide range of products that vary according to the region is milk

fermented with specific probiotic bacteria [4]. The concept of probiotic refers to microorganisms that, ingested in the right amount, can offer benefits to the health of their host, among which are protection against intestinal infections, stimulation of the immune system, and increase of milk digestibility, in addition to competing against pathogenic bacteria, allowing the proliferation of the normal intestinal microbiota [5]. Of the different species of probiotic microorganisms that are currently known, the most used in the food industry as part of the starter cultures belong to the group of lactic acid bacteria (LAB), more specifically, the genera *Lactobacillus*, *Lactococcus*, and *Bifidobacterium* [6]. However, for the culture to thrive as a probiotic, it must survive, develop, and remain viable in the medium during fermentation and storage time in concentrations close to 10^9 to 10^{12} CFU/mL [7].

The metabolic activity and the growth of certain strains of bacteria in the intestine are known to be stimulated by specific indigestible diet components [8,9]. These substances, called prebiotics, are available in nature as carbohydrates: fructooligosaccharides (FOS), galactooligosaccharides, lactulose, and fiber, among others. These compounds act as the primary energy source for the intestinal microbiota. In this way, the addition of prebiotics helps to improve the survival of beneficial microorganisms due to the symbiotic effect between them [10,11]. However, other ingredients, such as arabinoxylans and glucans, have prebiotic activity and have been studied more recently or are candidates and are in the process of demonstrating their effect in humans [12,13].

In this case is aguamiel, a liquid extracted from different magueys containing other carbohydrates such as fructose, glucose, sucrose, proteins, and minerals [14]. It is a source of prebiotics due to its fructan content and has the potential to be used in different branches of the food industry as a functional drink [15–17]. It is because of its composition that it is an excellent raw material to carry out fermentation [18,19], and being an aqueous medium with a large number of carbohydrates as substrates, it represents a favorable substrate for the growth of microorganisms and for the biotechnological production of high purity FOS [20–22]. This product has been used successfully to manufacture symbiotic foods such as fermented milk and cheese [18,19]. In addition, it has been associated with beneficial effects on human health in patients with irritable bowel syndrome [23,24].

Therefore, the objective of this research was to study the effect of aguamiel on changes in fermentation kinetics and the viability of *Lactiplantibacillus pentosus* ABHEAU-05 as a probiotic microorganism in enriched fermented milk, determining its ability to keep the probiotic viable in refrigerated storage as well as its proteolytic capacity.

2. Materials and Methods

2.1. Obtaining and Analysis of Aguamiel

Aguamiel was obtained from scraping maguey (*Agave salmiana*) in Zempoala, Hidalgo, Mexico. It was filtered and collected in 250 mL sterilized glass bottles. Then, a heat treatment was applied to the aguamiel at 90 °C for 15 min and stored at −20 °C before use. The carbohydrate concentration was determined following Moreno-Vilet et al. [25] methodology. An HPLC equipment (Agilent Technologies 1260 Ifinity) was occupied. A size exclusion column with a column guard (Ultrahydrogel DP, 7.8 × 300 mm, Waters, MA, USA) was used. At the same time, the mobile phase was water HPLC grade at a flow rate of 0.36 mL/min and a Light Scattering detector (Polymer Laboratories, Amherst, MA, USA). The elution medium was pre-degassed water HPLC grade (61.7 °C) at a flow rate of 0.36 mL/min. The run was carried out at a constant temperature of 75 °C, and the detector was maintained at a fogging temperature of 110 °C.

2.2. Adaptation of Lactiplantibacillus pentosus ABHEAU-05

This study used only *L. pentosus* ABHEAU-05 as a starter microorganism. The microorganism was isolated from a traditional Mexican drink (tepache), previously tested for its probiotic capacity, and phylogenetically identified with GeneBank accession number MK587617 [26]. It was stored frozen in 50% glycerol containment solution before use.

The microorganism was activated in the fermentation medium by inoculating 500 µL of the containment medium in a tube with Man Rogosa and Sharp (MRS) broth (propagation culture) and incubated at 37 °C for 24 h. A 12% *w/v* skimmed milk solution (Svelty Nestlé®, Nestlé, Vevey, Switzerland) was prepared, and 100 mL were placed in a previously sterilized Erlenmeyer flask, pasteurizing at 90 °C for 10 min. The milk solution was inoculated with 1 mL of the propagation culture and incubated at 37 °C for 24 h. The viable microorganisms (CFU/mL) were measured using plate count with an Agar-MRS medium. This was the starter culture.

2.3. Fermentation Kinetics

Fermentation was carried out in previously sterilized 250 mL Erlenmeyer flasks with screw caps. A 12% (*w/v*) solution of skimmed milk (Svelty Nestlé®) was prepared with sterilized water and with 2% of carbon source calculated from aguamiel analysis completing 100 mL. The solution was pasteurized at 90 °C for 15 min. Inulin was used as a control carbon source due to its wide use and recognition as a prebiotic used in different studies [18,19]. It was added to the control solution 2% agave inulin E-nature (Olifructine®, Innovation Naturals SAS, Roissy-en-France, France) with the following composition: polisaccharides > 10 units, 69.94%; polisaccharides from 2 to 10 units, 26.36%; and monosaccharides, 8.71% [25]. The systems were inoculated approximately with 10^6 CFU/mL of the previously adapted strain and incubated at 37 °C. Each fermentation system (control and understudy) was prepared in duplicate. Fermentations were stopped when a pH of 4.5 was reached, sampling every 2 h. The growth curve was performed by plate counting of the samples using Agar-MRS. The pH was determined, and the speed and generation time were calculated to establish the differences in the metabolism of microorganisms. The specific growth rate constant (µ) was calculated according to Equations (1) and (2) was used to determine the generation time (g). The initial concentration (N_0) was that corresponding to the beginning of the exponential phase (t_0), and the final (N_x) was that corresponding to the end of the exponential phase (t_x).

$$\text{Ln}\,(N_x) - \text{Ln}\,(N_0) = \mu\,(t_x - t_0) \quad (1)$$

$$g = \text{Ln}\,(2)/\mu \quad (2)$$

2.4. Proteolysis by Tri-Nitro Benzyl Sulfonic (TNBS)

The concentration of free amino groups was analyzed using the trinitrobenzylsulfonic acid (TNBS) method [27]. An aliquot of 5 mL of each fermentation system was taken every 2 h. After measuring the pH of each sample, they were centrifuged at 7800× *g* at 4 °C for 10 min in a centrifuge (Solbat for Eppendorf tubes). The supernatant was kept frozen at −14 °C for subsequent analysis. The concentration of free amino groups was determined according to a glycine standard curve from 0.05 mg/mL to 0.25 mg/mL. It was read at 340 nm in a spectrophotometer (GENESYS 10-VIS, Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.5. Evaluation of Refrigerated Fermented Milk

After fermentation (reaching a pH close to 4.5), the systems were stored at 4 °C for 21 days. Samples of 5 mL were taken at 7, 14, and 21 days. The samples were centrifuged in an Eppendorf centrifuge at 7800× *g* at 4 °C for 10 min. The following analyses were carried out: survival of *L. pentosus* ABHEAU-05, determination of free amino groups, and separation of peptides by polyacrylamide gel electrophoresis (SDS-PAGE). The analyses of free amino groups were carried out using the same technique used for the fermentation samples.

2.6. Separation of Hydrolyzed Milk Proteins by Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The separation of the hydrolyzed proteins after fermentation was carried out using the method proposed by Laemmli [28] with the modifications of González-Olivares et al. [27]. A Tris-Glycine gel was used with a T = 15%. A wide-range molecular weight standard (Bio-Rad, Berkeley, CA, USA) was used to determine molecular weights. The protein content of the samples was standardized to 150 ppm by analyzing with the Bradford method. Gels were stained with Comassie blue G-250 (Bio-Rad, Hercules, CA, USA) and analyzed with Gel-Doc software (Ez Imager #1708270, BioRad, Hercules, CA, USA).

2.7. Statistical Analysis

In all the experiments, triplicates were carried out, and the experimental data were submitted to an analysis of variance (ANOVA); the Tuckey method compared means with a significance level of 0.05. The program used for the analysis was NCSS-2007 (version: 07-1-15).

3. Results and Discussion

3.1. Aguamiel Analysis

The carbohydrates of the aguamiel were determined, and the compounds were identified according to the retention times of a standard curve of carbohydrate standards. The peaks corresponding to the polysaccharides of more than two units were observed between 13 and 14 min of retention. The retention time between 17 and 18 min reflected the presence of sucrose. This peak was the one that represented the highest concentration of all the separated carbohydrates. The final concentration of carbohydrates in aguamiel was 9.8% (*w/v*). Fructose and glucose reached 12.19% and 7.06%, respectively, while the sucrose concentration was 60%. In the end, the concentration of polysaccharides of more than two carbons was 20.75% (Table S1).

Moreno-Vilet et al. [25] mention that sucrose is the most abundant carbohydrate in aguamiel, used as raw material to produce tequila and pulque. However, the presence of monosaccharides is an essential factor in fermentation processes. Furthermore, aguamiel is rich in carbohydrates such as agavin or inulin. These carbohydrates have been considered prebiotic compounds [29].

3.2. Fermentation Kinetics

Figure 1 shows the graphs of the fermentation kinetics. No significant difference was established in the maximum concentration of microorganisms in both fermentations, even though the concentration was consistently higher for the system with aguamiel for up to 24 h. From that time, the concentrations were matched, and at 28 h, the system with inulin reached a concentration of 8.88 log CFU/mL, similar to that achieved in the system with aguamiel. Furthermore, this maximum increase was reached faster in the presence of aguamiel than in the control system. The highest value was approximated at 16 h of fermentation.

In contrast, in the system with inulin, it was a logarithmic cycle from the start of fermentation until hour 8, and from then until hour 28, the maximum cell concentration was found (8.66 log CFU/mL), which corresponded to the start of the stationary phase. Despite not presenting a significant difference between the maximum concentration obtained between the two systems, the speed to reach the stationary phase was faster in the system with aguamiel. The pH of 4.5 reached 18 h for the aguamiel fermentation and 36 h for the control fermentation. Despite the time difference, the growth was followed for 36 h, but the fermented milk was stored refrigerated once the pH of 4.5 was reached (Figure S1).

Aguamiel had already been used as an ingredient added to milk for fermentation. Jaimez-Ordaz et al. [18] demonstrated that using aguamiel causes a faster drop in pH than milk fermentations added with inulin. This could be due to the composition of the aguamiel used, which had a higher amount of FOS. In addition, it is known that the decrease in pH

in fermentation systems such as the one in this study is also related to the production of short-chain organic acids [30].

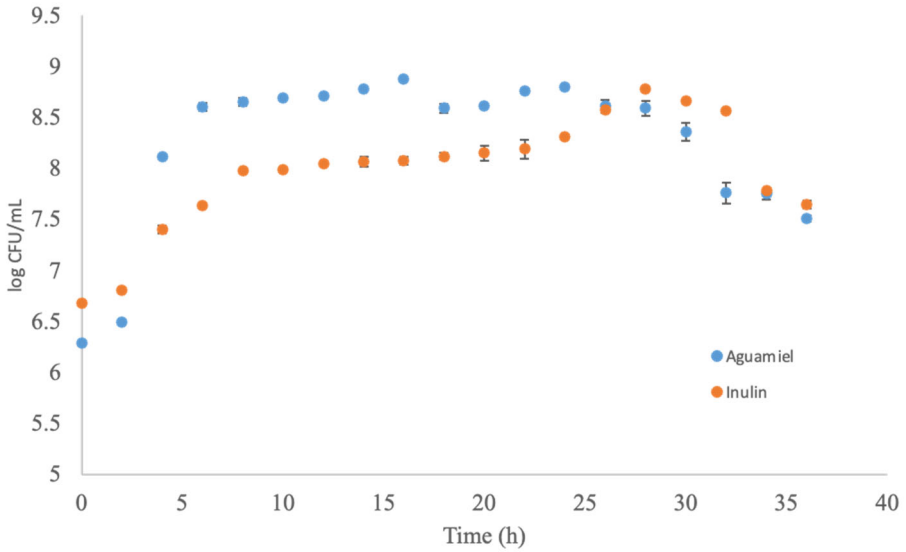

Figure 1. Growth curves of *Lactiplantibacillus pentosus ABHEAU-05* in fermented milk enriched with • aguamiel and • inulin.

These data reveal the influence that aguamiel has on the fermentation rate. Despite the changes in the growth of the microorganism, it was possible to differentiate the adaptation phase in neither of the two systems. This may be because the composition of the medium provided adequate substrates to be consumed immediately by *L. pentosus* ABHEAU-05. With these data, the kinetic calculations of the fermentation were made. The specific growth rate (μ) for the exponential phase and the doubling time (g) were determined (Table 1).

Table 1. Kinetic parameters of *L. pentosus* ABHEAU-05 growth during milk fermentation with aguamiel and inulin.

Carbon Source	μ (h^{-1})	g (h)
Aguamiel	^{a} 0.98 ±0.04	^{a} 0.71 ±0.0
Inulin	^{b} 0.43 ±0.03	^{b} 1.61 ±0.01

All results are expressed as the mean ± standard deviation (n = three replicates). Different letters denote significant differences within the columns ($p < 0.05$).

The ability to adapt is easy to distinguish with these data. In the medium with aguamiel, better kinetic parameters were obtained compared to that of agave inulin since, during the fermentation process, the strain of *L. pentosus* ABHEAU-05 reached a higher speed (h^{-1}) and, therefore, a minor doubling time (g). This reflected a higher cell concentration during the exponential phase.

In two different studies carried out by Gámez et al. [31] and Concha et al. [32] on the kinetic behavior of *Lactobacillus* species with and without a prebiotic source, the results obtained in the kinetic parameters (μ = 0.975 h^{-1}, g = 0.70 h and μ = 0.53 h^{-1}, g = 1.28 h; respectively) showed a contrast between the metabolic capacity of the bacteria. This capacity is favored using prebiotic substances because these substrates tend to the growth of microorganisms in a different way.

The most determining factors in the development of probiotic LAB in prebiotics are the degree of polymerization, the number of repeating units in carbohydrates, and the chemical structure [33]. Microorganisms' development and use of the substrate also depend on the availability of fermentable sugars in the medium [34]. Thus, a combination of carbohydrates in the culture improves the growth of bacteria [35–37].

Aguamiel used in this study showed a remarkable ability to promote LAB growth than inulin due to its composition in monosaccharides, disaccharides, and oligosaccharides. LAB will begin to consume the polysaccharides in a medium-low in simple carbohydrates, such as inulin [38]. This action leads to higher energy expenditure and a slowdown in the fermentation process because the degree of polymerization and the branching of the molecule will affect the degradation of the oligo and polysaccharides. Previous studies using aguamiel as a carbon source for developing LAB in different dairy products have shown its ability to maintain viability despite such a high concentration of sucrose [18,19].

3.3. Determination of Free Amino Groups during Fermentation

The concentration of free amino groups was determined during the fermentation time for each system and each carbon source (Table 2). To analyze the proteolytic capacity of each fermentation, samples were taken from the middle start and end of each fermentation within its logarithmic phase. That is why the fermentation with aguamiel samples was carried out at 0, 8, and 18 h. In the case of fermentation with inulin, samples were taken at 0, 18, and 36 h. The initial concentration of free amino groups in the fermentation carried out with inulin was 182.33 mg/L, reaching a final value of 113.72 mg/L at 36 h of fermentation. However, the highest value was reached at 18 h (263.44 mg/L). After this time, the decrease in concentration began. In the case of the system with aguamiel, the concentration of free amino groups increased only during the first eight hours of fermentation, reaching a maximum value of 255.56 mg/L. The final concentration was 162.05 mg/L at 18 h. When comparing the results obtained, it was observed that the concentration of free amino groups was higher in the system with inulin at the beginning and after 18 h of the fermentation process.

Table 2. Concentration of free amino groups during fermentation with *L. pentosus* ABHEAU-05 of milk enriched with aguamiel and inulin.

Stage of Fermentation	Amino Group Concentration Aguamiel (mg/L)	Stage of Fermentation	Amino Group Concentration Inulin (mg/L)
Beginning (0 h)	^{a} 156.78 $^{\pm 3.14}$	Beginning (0 h)	^{a} 182.33 $^{\pm 6.66}$
Middle (8 h)	^{b} 255.56 $^{\pm 4.23}$	Middle (18 h)	^{b} 263.44 $^{\pm 0.98}$
End (18 h)	^{a} 162.05 $^{\pm 7.01}$	End (36 h)	^{c} 113.72 $^{\pm 5.39}$

All results are expressed as the mean ± standard deviation (n = three replicates). Different letters denote significant differences within the columns ($p < 0.05$).

LABs require amino acids to grow, so protein hydrolysis during fermentation is significant since it will directly affect metabolism. However, the proteolytic system of each strain is different from others according to the peptidases that each microorganism possesses [39]. In the specific case of *L. pentosus* ssp, the activity of dipeptidases and aminopeptidases has been reported, which act as releasers of amino acids with terminal carbons in the protein sequence [40].

3.4. Metabolism during Refrigeration

3.4.1. Viability

At the end of fermentation, the viable count was similar in both systems (8.68 log CFU/mL in aguamiel and 8.65 log CFU/mL with inulin). Thus, the initial concentration was comparable at the beginning of refrigerated storage. The counts obtained at the end of storage were 8.47 log CFU/mL with aguamiel and 8.30 CFU/mL with inulin. No statistically significant differences were found between the two fermentations. The evolution in the survival of *L. pentosus* ABHEAU-05 during the three weeks of storage can be seen in Table 3.

Table 3. Survival of *L. pentosus* ABHEAU-05 at the end of fermentation and refrigerated storage at 4 °C in fermented milk enriched with aguamiel and inulin.

Refrigeration Time (Week)	Aguamiel (log CFU/mL)	Inulin (log CFU/mL)
End of fermentation	^{a} 8.68 ±0.04	^{a} 8.65 ±0.01
1	^{a} 8.65 ±0.04	^{a} 8.62 ±0.02
2	^{b} 8.58 ±0.02	^{b} 8.46 ±0.02
3	^{b} 8.47 ±0.05	^{c} 8.30 ±0.07

All results are expressed as the mean ± standard deviation (n = three replicates). Different letters denote significant differences within the columns ($p < 0.05$).

Similar concentrations of viable cells were observed in studies carried out in fermented milk without prebiotics by Nighswonger et al. [41] and Znamirowska et al. [42] during 15 and 21 days of refrigerated storage, respectively. However, a two-log cycle decrease was observed in these studies, which is attributed to the lack of a prebiotic in the medium.

The decrease in bacterial concentration during refrigerated storage after fermentation is mainly related to two factors. First, LAB is exposed to a temperature below their growth optimum, which affects the stability of their membrane, leading to alterations in cellular processes such as cell division, metabolism, and transport. On the other hand, the acidity in the medium is the second responsible factor since when the acids that pass through the membrane dissociate, the intracellular pH decreases, which reduces enzymatic activity, and proteins and DNA are damaged, affecting duplication [43]. In this sense, LAB can quickly adapt to temperature changes. Van de Guchte et al. [43] demonstrated that *Lactococcus lactis* sp., *Streptococcus thermophilus*, *Lactobacillus acidophilus*, and *Lactobacillus plantarum* continue to grow at a reduced rate after a 20 °C change below the optimum growth temperature.

In addition, the presence of a prebiotic in fermented milk favors the growth and maintenance of the viability of probiotics during refrigerated storage [18,44]. These studies aim to keep probiotics at recommended consumption levels. The concentrations obtained in this research are among the necessary values that foods must contain to be considered probiotics and were maintained throughout the refrigerated storage time (>10^8 CFU/mL). According to Olvera-Rosales et al. [9], the microorganisms must be viable in the food in concentrations of 10^6–10^{11} CFU/mL during the shelf life.

3.4.2. Production of Free Amino Groups

The initial concentration of free amino groups is considered the concentration at the end of fermentation (FF); for this reason, both systems started with different concentrations (Table 4).

Table 4. Concentration of free amino groups during refrigerated storage at 4 °C in fermented milk enriched with aguamiel and inulin.

Refrigeration Time (Week)	Aguamiel (mg/L)	Inulin (mg/L)
End of fermentation	^{a} 162.05 ±0.01	^{a} 113.72 ±5.74
1	^{b} 203.890 ±0.01	^{b} 206.81 ±9.20
2	^{b} 212.97 ±0.01	^{b} 208.58 ± 0.60
3	^{b} 215.87 ±0.008	^{b} 209.97 ±1.45

All results are expressed as the mean ± standard deviation (n = three replicates). Different letters denote significant differences within the columns ($p < 0.05$).

In the first week, the initial concentration in the fermented milk with inulin was 206.81 mg/L. The data obtained show a gradual increase throughout the storage time until reaching 209.7 mg/L in the last week. On the other hand, in the system with aguamiel, the concentration in the first week was 203.76 mg/L, a lower value than that obtained with inulin at the same time. Between the first and second week, there was a more notice-

able increase in concentration (212.84 mg/L), and at the end of the study, the maximum concentration was 215.87 mg/L. However, no significant difference was found between both systems.

Even though proteolysis is necessary for the survival of microorganisms, in refrigeration, this activity decreases but remains active [45], preserving only the production of free amino groups to maintain their dormancy within the food [46,47]. Liu et al. [48] describe one of the mechanisms some probiotic bacteria have adapted when exposed to a downward change in temperature called the cold shock response. In this mechanism, various proteins are synthesized to maintain the membrane's fluidity by increasing the proportion of shorter proteins, including low molecular weight proteins, thereby improving the process of cell reproduction.

3.4.3. Identification of Protein Hydrolysis by SDS-PAGE

Figure 2 shows the electrophoresis gels of the fermented milk stored under refrigeration for each carbon source. Bands with molecular weights lower than 14.4 kDa were found in all the lanes corresponding to each of the weeks of the analysis. As observed, there is an accumulation of peptides with a molecular weight of less than 6.5 kDa. In addition, a decrease in the concentration of milk proteins is observed, including those of caseins and the main whey proteins (21.5 and 14.4 kDa). This accumulation of peptides is important because some bioactive peptides can be found among them [40].

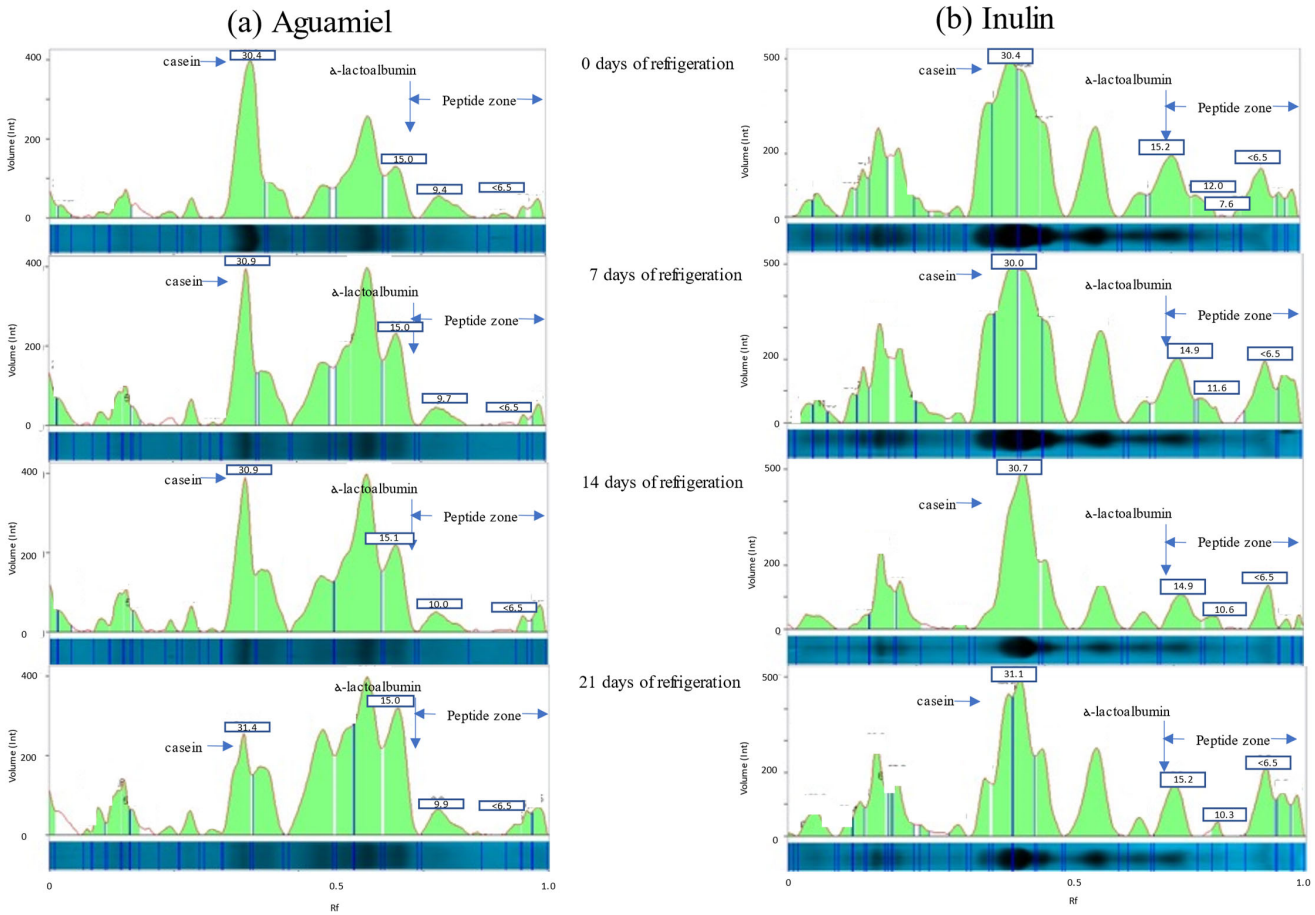

Figure 2. Peptide separation by SDS-PAGE of fermented milk enriched with (**a**) aguamiel and (**b**) inulin, refrigerated at 4 °C for 21 days.

As in the analysis of free amino groups, the production of low molecular weight peptides is a marker of the metabolic activity of viable cells in the medium. González-Olivares et al. [27,49] reported low molecular weight peptides produced during

the refrigeration of commercial fermented milk. In addition, it has been determined that these peptides produced during fermentation could have bioactive potential [50]. Additionally, it has been found that the biological activity of peptides is also increased during refrigerated storage of fermented milk [51,52]. This must be related to the proteolytic activity and the enzymes released from the cells lysed in the medium [41].

Although peptides smaller than 6.5 kDa are observed in both experiments, the concentration is higher in those separated in milk enriched with inulin. However, as can be seen, the concentration of high molecular weight milk proteins such as caseins decreased more in milk enriched with aguamiel. In the case of milk with inulin, the apparent concentrations of both caseins and whey proteins appear to be with minimal changes. This means that, since the proteolytic system is a cascade system [39], the production of low molecular weight peptides comes from peptides of intermediate weight in the case of milk with inulin. In contrast, in the case of milk with aguamiel, the peptides could go directly from caseins since the concentration of whey proteins seems to maintain their concentration. It is known that bioactive peptides, especially with antihypertensive capacity, come from sequences released from caseins, mainly ß-casein [50].

L. pentosus has dipeptidase activity that can hydrolyze proteins to obtain Leu-Leu, Phe-Ala, Tyr-Leu Lys-Leu, Ala-Ala, and Leu-Gly bonds, among other dipeptides. Aminopeptidase activity has also been reported for Ala, Lys, Pro, and Leu, which act on the ends of the chains, releasing the terminal amino acid [40].

It has been reported that probiotic bacteria generally show a population decline due to low storage temperatures. This is caused by transport changes in the cell membrane that can sometimes suffer irreversible damage, changing the entire nutrient transport scheme [53,54]. However, the presence of prebiotics accounts for their importance in the containment of viable cells in dairy products [55]. On the other hand, the acidification of the medium is a determining factor in the decrease of viable cells due to the modifications in the expression of proteins necessary for the transport of nutrients. Studies have revealed the importance of these structural modifications of probiotic cells for their ability to survive both cold stress and pH changes [56]. In our study, microorganisms were contained by not observing a significant difference in concentration during storage. According to the results and bibliographic reports [55], this effect is attributable to aguamiel [18,19].

Despite the use of aguamiel in fermented milk and fermented cheeses, this study represents the first in the application of *L. pentosus* ABHEAU-05 determining its ability to relate symbiotically with a product such as aguamiel as a prebiotic for the design of a symbiotic food. In addition, aguamiel could be a potential prebiotic, and its derived products, such as aguamiel syrup, have shown sufficient scientific evidence as a beneficial material for human health [57].

4. Conclusions

Aguamiel synergizes with *L. pentosus* ABHEAU-05, promoting its growth and lowering the pH during fermentation, like inulin. In addition, the survival of the microorganism during refrigerated storage was favored even without having ruled out the effect of lactose or the monosaccharides present in aguamiel. Further studies on the effect of monosaccharides and disaccharides in aguamiel on the survival of probiotics are needed. However, this results in a direct relationship between the presence of aguamiel and the activation of the proteolytic system during the fermentation process. Because the experiments were compared with a control with a prebiotic known as inulin and they were carried out under equal conditions, the viability of *L. pentosus* ABHEAU-05 is an effect of the presence of aguamiel, which promotes the production of low molecular weight peptides even under refrigeration. That is why using aguamiel with *L. pentosus* ABHEAU-05 could be an alternative to producing symbiotic foods, mainly due to the prebiotic potential of a natural product such as agave pulquero aguamiel.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9090841/s1, Figure S1: Evolution of pH during milk fermentation with *Lactobacillus pentosus* ABHEAU-05 in systems enriched with aguamiel and inulin; Table S1: Results of the carbohydrates separation of aguamiel by -SEC-HPLC.

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References

1. Granato, D.; Barba, F.J.; Bursać Kovačević, D.; Lorenzo, J.M.; Cruz, A.G.; Putnik, P. Functional foods: Product development, technological trends, efficacy testing, and safety. *Annu. Rev. Food Sci. Technol.* **2020**, *11*, 93–118. [CrossRef]
2. Ari, S. Studies on functional foods in Japan—State of the art. *Biosci. Biotechnol. Biochem.* **1996**, *60*, 9–15. [CrossRef]
3. Domínguez, D.L.; Fernández, R.V.; Cámara, M. An international regulatory review of food health-related claims in functional food products labeling. *J. Funct. Foods* **2020**, *68*, 103896. [CrossRef]
4. Savaiano, D.A.; Hutkins, R.W. Yogurt, cultured fermented milk, and health: A systematic review. *Nutr. Rev.* **2021**, *79*, 599–614. [CrossRef] [PubMed]
5. Zendeboodi, F.; Khorshidian, N.; Mortazavian, A.M.; G da Cruz, A. Probiotic: Conceptualization from a new approach. *Curr. Opin. Food Sci.* **2020**, *32*, 103–123. [CrossRef]
6. Chen, W.; Hang, F. *Lactic Acid Bacteria Starter. Lactic Acid Bacteria: Bioengineering and Industrial Applications*; Springer: Berlin/Heidelberg, Germany, 2019; pp. 93–143.
7. Palanivelu, J.; Thanigaivel, S.; Vickram, S.; Dey, N.; Mihaylova, D.; Desseva, I. Probiotics in Functional Foods: Survival Assessment and Approaches for Improved Viability. *Appl. Sci.* **2022**, *12*, 455. [CrossRef]
8. Aoun, A.; Darwish, F.; Hamod, N. The influence of the gut microbiome on obesity in adults and the role of probiotics, prebiotics, and synbiotics for weight loss. *Prev. Nutr. Food Sci.* **2020**, *25*, 113–123. [CrossRef] [PubMed]
9. Olvera-Rosales, L.-B.; Cruz-Guerrero, A.-E.; Ramírez-Moreno, E.; Quintero-Lira, A.; Contreras-López, E.; Jaimez-Ordaz, J.; Castañeda-Ovando, A.; Añorve-Morga, J.; Calderón-Ramos, Z.-G.; Arias-Rico, J.; et al. Impact of the Gut Microbiota Balance on the Health–Disease Relationship: The Importance of Consuming Probiotics and Prebiotics. *Foods* **2021**, *10*, 1261. [CrossRef] [PubMed]
10. Swanson, K.S.; Gibson, G.R.; Hutkins, R.; Reimer, R.A.; Reid, G.; Verbeke, K.; Scott, K.P.; Holscher, D.H.; Azad, M.B.; Delzenne, N.M.; et al. The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of synbiotics. *Nat. Rev. Gastroenterol. Hepatol.* **2020**, *17*, 687–701. [CrossRef]
11. Yilmaz, S.; Yilmaz, E.; Dawood, M.A.; Ringø, E.; Ahmadifar, E.; Abdel-Latif, H.M. Probiotics, prebiotics, and synbiotics used to control vibriosis in fish: A review. *Aquaculture* **2022**, *547*, 737514. [CrossRef]
12. Fan, S.; Zhang, Z.; Zhao, Y.; Daglia, M.; Zhang, J.; Zhu, Y.; Bai, J.; Zhu, L.; Xiao, X. Recent advances in targeted manipulation of the gut microbiome by prebiotics: From taxonomic composition to metabolic function. *Curr. Opin. Food Sci.* **2022**, *49*, 100959. [CrossRef]
13. Pang, J.; Zhang, Y.; Tong, X.; Zhong, Y.; Kong, F.; Li, D.; Liu, X.; Qiao, Y. Recent Developments in Molecular Characterization, Bioactivity, and Application of Arabinoxylans from Different Sources. *Polymers* **2023**, *15*, 225. [CrossRef]
14. Ortiz-Basurto, R.I.; Pourcelly, G.; Doco, T.; Williams, P.; Dornier, M.; Belleville, M.P. Analysis of the main components of the aguamiel produced by the maguey-pulquero (*Agave mapisaga*) throughout the harvest period. *J. Agric. Food Chem.* **2008**, *56*, 3682–3687. [CrossRef]
15. Romero-López, M.R.; Osorio-Díaz, P.; Flores-Morales, A.; Robledo, N.; Mora-Escobedo, R. Chemical composition, antioxidant capacity and prebiotic effect of aguamiel (*Agave atrovirens*) during in vitro fermentation. *Rev. Mex. Ing. Quim.* **2015**, *14*, 281–292.
16. Villarreal-Morales, S.L.; Muñiz-Márquez, D.B.; Michel-Michel, M.; González-Montemayor, Á.M.; Escobedo-García, S.; Salas-Tovar, J.A.; Flores, C.; Rodríguez-Herrera, R. Aguamiel a fresh beverage from *Agave* spp. sap with functional properties. In *Natural Beverages*; Academic Press: Cambridge, MA, USA, 2019; pp. 179–208.

17. Peralta-García, I.; González-Muñoz, F.; Rodríguez-Alegría, M.E.; Sánchez-Flores, A.; López-Munguía, A. Evolution of fructans in aguamiel (*Agave sap*) during the plant production lifetime. *Front. Nutr.* **2020**, *7*, 566950. [CrossRef] [PubMed]
18. Jaimez-Ordaz, J.; Martinez-Ramirez, X.; Cruz-Serrano, A.E.; Contreras-López, E.; Ayala-Nino, A.; Castro-Rosas, J.; González-Olivares, L.G. Survival and proteolytic capacity of probiotics in a fermented milk enriched with agave juice and stored in refrigeration. *Food Sci. Technol.* **2019**, *39*, 188–194. [CrossRef]
19. Ramírez-Godínez, J.; Gutiérrez-Rodríguez, J.F.; Contreras-López, E.; Rodríguez-Serrano, G.M.; Castañeda-Ovando, A.; Jaimez-Ordaz, J.; González-Olivares, L.G. Agave juice improves survival and proteolytic activity of *Lactobacillus rhamnosus* GG during ripening of semi-ripened mexican cheese. *Food Sci. Technol.* **2021**, *42*, e30820. [CrossRef]
20. Escalante, A.; Giles-Gómez, M.; Esquivel-Flores, G.; Matus Acuña, V.; Moreno-Terrazas, R.; López-Munguía, A.; Lappe-Oliveras, P. Pulque fermentation. In *Handbook of Plant-Based Fermented Food and Beverage Technology*; Routledge: London, UK, 2012; Volume 2, pp. 691–706.
21. Escalante, A.; López Soto, D.R.; Velázquez Gutiérrez, J.E.; Giles-Gómez, M.; Bolívar, F.; López-Munguía, A. Pulque, a traditional Mexican alcoholic fermented beverage: Historical, microbiological, and technical aspects. *Front. Microbiol.* **2016**, *7*, 1026. [CrossRef] [PubMed]
22. de la Rosa, O.; Flores-Gallegos, A.C.; Muñíz-Márquez, D.; Contreras-Esquivel, J.C.; Teixeira, J.A.; Nobre, C.; Aguilar, C.N. Successive Fermentation of Aguamiel and Molasses by *Aspergillus oryzae* and *Saccharomyces cerevisiae* to Obtain High Purity Fructooligosaccharides. *Foods* **2022**, *11*, 1786. [CrossRef]
23. Carrillo-López, A.; Silos-Espino, H.; Flores-Benitez, S.; Espinoza-Sánchez, E.A.; Ornelas-Tavares, J.R.; Flores-Chávez, L.; Tovar-Robles, C.; Méndez-Gallegos, J.; Rossel-Kipping, D. Some evidences on effect of intake aguamiel (*Agave sap*). *Sustain. Agric. Res.* **2016**, *5*, 49–55. [CrossRef]
24. Cervantes-Elizarrarás, A.; Cruz-Cansino, N.d.S.; Ramírez-Moreno, E.; Vega-Sánchez, V.; Velázquez-Guadarrama, N.; Zafra-Rojas, Q.Y.; Piloni-Martini, J. In vitro probiotic potential of lactic acid bacteria isolated from aguamiel and pulque and antibacterial activity against pathogens. *Appl. Sci.* **2019**, *9*, 601. [CrossRef]
25. Moreno-Vilet, L.; Bostyn, S.; Flores-Montaño, J.L.; Camacho-Ruiz, R.M. Size-exclusion chromatography (HPLC-SEC) technique optimization by simplex method to estimate molecular weight distribution of agave fructans. *Food Chem.* **2017**, *237*, 833–840. [CrossRef] [PubMed]
26. Escobar-Ramírez, M.C.; Jaimez-Ordaz, J.; Escorza-Iglesias, V.A.; Rodríguez-Serrano, G.M.; Contreras-López, E.; Ramírez-Godínez, J.; Castañeda-Ovando, A.; Morales-Estrada, A.I.; Felix-Reyes, N.; González-Olivares, L.G. *Lactobacillus pentosus* ABHEAU-05: An in vitro digestion resistant lactic acid bacterium isolated from a traditional fermented Mexican beverage. *Rev. Argent. Microbiol.* **2020**, *52*, 305–314. [CrossRef] [PubMed]
27. González-Olivares, L.G.; Añorve-Morga, J.; Castañeda-Ovando, A.; Contreras-López, E.; Jaimez-Ordaz, J. Peptide separation of commercial fermented milk during refrigerated storage. *Food Sci. Technol.* **2014**, *34*, 674–679. [CrossRef]
28. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685. [CrossRef] [PubMed]
29. Fernández-Lainez, C.; López-Velázquez, G.; de Vos, P. Health benefits of Inulin and Agavin-type Fructans in Food: Impact on Microbiota, Immune and Gut Barrier Function. In *The Book of Fructans*; Academic Press: Cambridge, MA, USA, 2023; pp. 211–234.
30. Zalán, Z.; Hudáček, J.; Štětina, J.; Chumchalová, J.; Halász, A. Production of organic acids by *Lactobacillus strains* in three different media. *Eur. Food Res. Technol.* **2010**, *230*, 395–404. [CrossRef]
31. Gámez, H.J.; Ramírez, C.; Aguirre, D. Cinética de fermentación de *Lactobacillus plantarum* en un medio de cultivo enriquecido como potencial probiótico. *Rev. Vet. Zootec.* **2013**, *7*, 37–53.
32. Concha, J.L.H.; Agudelo, C.; Ortega, R. Determinación de parámetros cinéticos de dos inóculos lácticos: *Lactobacillus plantarum* A6 y bacterias ácido lácticas de yogurt. *Biotecnol. Sect. Agropecu. Agroind.* **2010**, *8*, 8–16.
33. Mueller, M.; Reiner, J.; Fleischhacker, L.; Viernstein, H.; Loeppert, R.; Praznik, W. Growth of selected probiotic strains with fructans from different sources relating to degree of polymerization and structure. *J. Funct. Foods* **2016**, *24*, 264–275. [CrossRef]
34. Böger, M.; Van Leeuwen, S.S.; Lammerts van Bueren, A.; Dijkhuizen, L. Structural identity of galactooligosaccharide molecules selectively utilized by single cultures of probiotic bacterial strains. *J. Agric. Food Chem.* **2019**, *67*, 13969–13977. [CrossRef]
35. Kneifel, W. In vitro growth behaviour of probiotic bacteria in culture media with carbohydrates of prebiotic importance. *Microb. Ecol. Health Dis.* **2000**, *12*, 27–34.
36. Di Lodovico, S.; Gasparri, F.; Di Campli, E.; Di Fermo, P.; D'Ercole, S.; Cellini, L.; Di Giulio, M. Prebiotic combinations effects on the colonization of Staphylococcal skin strains. *Microorganisms* **2021**, *9*, 37. [CrossRef] [PubMed]
37. Oluwatosin, S.O.; Tai, S.L.; Fagan-Endres, M.A. Sucrose, maltodextrin and inulin efficacy as cryoprotectant, preservative and prebiotic–towards a freeze dried *Lactobacillus plantarum* topical probiotic. *Biotechnol. Rep.* **2022**, *33*, e00696. [CrossRef] [PubMed]
38. Kaplan, H.; Hutkins, R.W. Fermentation of fructooligosaccharides by lactic acid bacteria and bifidobacteria. *Appl. Environ. Microbiol.* **2000**, *66*, 2682–2684. [CrossRef] [PubMed]
39. Rodríguez-Serrano, G.M.; García-Garibay, M.; Cruz-Guerrero, A.E.; Gómez-Ruiz, L.; Ayala-Niño, A.; Castañeda-Ovando, A.; González-Olivares, L.G. Proteolytic System of *Streptococcus thermophilus*. *J. Microbiol. Biotechnol.* **2018**, *28*, 1581–1588. [CrossRef]
40. Toldrá, F.; Reig, M.; Aristoy, M.C.; Mora, L. Generation of bioactive peptides during food processing. *Food Chem.* **2018**, *267*, 395–404. [CrossRef] [PubMed]

41. Nighswonger, B.D.; Brashears, M.M.; Gilliland, S.E. Viability of *Lactobacillus acidophilus* and *Lactobacillus casei* in fermented milk products during refrigerated storage. *J. Dairy Sci.* **1996**, *79*, 212–219. [CrossRef]
42. Znamirowska, A.; Szajnar, K.M.; Pawlos, M. Probiotic Fermented Milk with Collagen. *Dairy* **2020**, *1*, 126–134. [CrossRef]
43. Van De Guchte, M.; Serror, P.; Chervaux, C.; Smokvina, T.; Ehrlich, S.D.; Maguin, E. Stress responses in lactic acid bacteria. In *Lactic Acid Bacteria: Genetics, Metabolism and Applications, Proceedings of the Seventh Symposium on Lactic Acid Bacteria: Genetics, Metabolism and Applications, Egmond aan Zee, The Netherlands, 1–5 September 2002*; Springer: Dordrecht, The Netherlands, 2002; pp. 187–216.
44. Nielsen, M.S.; Martinussen, T.; Flambard, B.; Sørensen, K.I.; Otte, J. Peptide profiles and angiotensin-I-converting enzyme inhibitory activity of fermented milk products: Effect of bacterial strain, fermentation pH, and storage time. *Int. Dairy J.* **2009**, *19*, 155–165. [CrossRef]
45. Shi, J.; Luo, Y.; Xiao, Y.; Li, Z.; Xu, Q.; Yao, M. Effects of fermentation by *Lactobacillus casei* on the antigenicity and allergenicity of four bovine milk proteins. *Int. Dairy J.* **2014**, *35*, 75–80. [CrossRef]
46. Bu, G.; Luo, Y.; Zhang, Y.; Chen, F. Effects of fermentation by lactic acid bacteria on the antigenicity of bovine whey proteins. *J. Sci. Food Agric.* **2010**, *90*, 2015–2020. [CrossRef] [PubMed]
47. Olvera-Rosales, L.B.; Pérez-Escalante, E.; Castañeda-Ovando, A.; Contreras-López, E.; Cruz-Guerrero, A.E.; Regal-López, P.; Cardelle-Cobas, A.; González-Olivares, L.G. ACE-Inhibitory Activity of Whey Proteins Fractions Derived of Fermentation by *Lacticaseibacillus rhamnosus* GG and *Streptococcus thermophilus* SY-102. *Foods* **2023**, *12*, 2416. [CrossRef] [PubMed]
48. Liu, S.; Ma, Y.; Zheng, Y.; Zhao, W.; Zhao, X.; Luo, T.; Zhang, J.; Yang, Z. Cold-stress response of probiotic *Lactobacillus plantarum* K25 by iTRAQ proteomic analysis. *J. Microbiol. Biotechnol.* **2020**, *30*, 187. [CrossRef] [PubMed]
49. González-Olivares, L.G.; Jiménez-Guzmán, J.; Cruz-Guerrero, A.; Rodríguez-Serrano, G.; Gómez-Ruiz, L.; García-Garibay, M. Bioactive peptides released by lactic acid bacteria in commercial fermented milks. *Rev. Mex. Ing. Quím.* **2011**, *10*, 179–188.
50. Sebastián-Nicolas, J.L.; Contreras-López, E.; Ramírez-Godínez, J.; Cruz-Guerrero, A.E.; Rodríguez-Serrano, G.M.; Añorve-Morga, J.; Jaimez-Ordaza, J.; Castañeda-Ovando, A.; Pérez-Escalante, E.; Ayalo-Niño, A.; et al. Milk fermentation by Lacticaseibacillus rhamnosus GG and *Streptococcus thermophilus* SY-102: Proteolytic profile and ace-inhibitory activity. *Fermentation* **2021**, *7*, 215. [CrossRef]
51. Donkor, O.N.; Henriksson, A.; Singh, T.K.; Vasiljevic, T.; Shah, N.P. ACE-inhibitory activity of probiotic yoghurt. *Int. Dairy J.* **2007**, *17*, 1321–1331. [CrossRef]
52. Moslehishad, M.; Ehsani, M.R.; Salami, M.; Mirdamadi, S.; Ezzatpanah, H.; Naslaji, A.N.; Moosavi-Movahedi, A.A. The comparative assessment of ACE-inhibitory and antioxidant activities of peptide fractions obtained from fermented camel and bovine milk by *Lactobacillus rhamnosus* PTCC 1637. *Int. Dairy J.* **2013**, *29*, 82–87. [CrossRef]
53. Mbye, M.; Baig, M.A.; AbuQamar, S.F.; El-Tarabily, K.A.; Obaid, R.S.; Osaili, T.M.; Al-Nabulsi, A.A.; Turner, M.S.; Shah, N.P.; Ayyash, M.M. Updates on understanding of probiotic lactic acid bacteria responses to environmental stresses and highlights on proteomic analyses. *Compr. Rev. Food Sci. Food. Saf.* **2020**, *19*, 1110–1124. [CrossRef]
54. Fonseca, F.; Pénicaud, C.; Tymczyszyn, E.E.; Gómez-Zavaglia, A.; Passot, S. Factors influencing the membrane fluidity and the impact on production of lactic acid bacteria starters. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 6867–6883. [CrossRef]
55. Chávez de la Vega, M.I.; Alatorre-Santamaría, S.; Gómez-Ruiz, L.; García-Garibay, M.; Guzmán-Rodríguez, F.; González-Olivares, L.G.; Cruz-Guerrero, A.E.; Rodríguez-Serrano, G.M. Influence of Oat β-Glucan on the Survival and Proteolytic Activity of *Lactobacillus rhamnosus* GG in Milk Fermentation: Optimization by Response Surface. *Fermentation* **2021**, *7*, 210. [CrossRef]
56. Adamberg, K.; Kask, S.; Laht, T.M.; Paalme, T. The effect of temperature and pH on the growth of lactic acid bacteria: A pH-auxostat study. *Int. J. Food Microbiol.* **2003**, *85*, 171–183. [CrossRef] [PubMed]
57. Castañeda-Ovando, A.; Moreno-Vilet, L.; Jaimez-Ordaz, J.; Ramírez-Godínez, J.; Pérez-Escalante, E.; Cruz-Guerrero, A.E.; Contreras-López, E.; Alatorre-Santamaría, S.A.; Guzmán-Rodríguez, F.J.; González-Olivares, L.G. Aguamiel syrup as a technological diversification product: Composition, bioactivity and present panorama. *Future Foods* **2023**, *8*, 100249. [CrossRef]