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Exploring the Potential of Halloumi Second Cheese Whey for the Production of Lactic Acid Cultures for the Dairy Industry

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Abstract: Production of halloumi cheese in Cyprus has increased rapidly over the last decade since the popularity of the cheese is growing. This results in excess production of whey streams that pose serious environmental concerns and are difficult to manage. In our study, the poorly investigated second cheese whey (SCW) generated after the extraction of anari, a by-product of halloumi cheese manufacture, was examined for dairy industry lactic acid bacteria (LAB) culture production. The LAB studied were all initially isolated from SCW. These LAB were molecularly identified and characterized in an attempt to explore their potential use as starter cultures for the dairy industry. A total of 11 Gram-positive and catalase-negative isolates were identified, belonging to four different species/subspecies: *Lactobacillus delbrueckii* subsp. *lactis*, *Lactobacillus delbrueckii* subsp. *jakobsenii*, *Lactobacillus leichmannii* and *Lactobacillus crispatus*. Lactose/galactose utilization tests demonstrated species-specific differences in galactose and lactose catabolism. Interestingly, culturing the selected isolates in SCW supplemented with skimmed milk (30% total solids) improved their freeze-drying tolerance (75–91% survival rate depending on the species). Moreover, isolates in vacuum-packed powders maintained viability and metabolic activity over 3-month storage at 4 °C.

Keywords: halloumi cheese; second cheese whey; lactic acid bacteria; 16S rRNA gene sequencing; galactose catabolism; lactose catabolism; freeze-drying; storage

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1. Introduction

Halloumi cheese is a traditional and unique cheese of Cyprus. The definition and standards for the traditional product have been established by the Cyprus Ministry of Commerce and Industry [1]. It is widely popular in Cyprus and other countries of the eastern Mediterranean and, over the past decade, the product has gained global recognition. Noticeably, the exports of halloumi from Cyprus over the past five years have shown a steady annual increase of 20–22%. In the 2017–2019 period, the volume of exports jumped from 23,431 tons in 2017 to 33,672 tonnes in 2019, marking an increase of 43% in just three years [2]. This increase in production results in excess whey production, from which the whey cheese anari is extracted. The liquid stream that is left over after the whey cheese separation is referred to as second cheese whey (SCW) and is handled by most halloumi cheese producers in Cyprus (if not all) as a typical waste stream. This involves the storage of the SCW in silos for a period of up to 48 h at a decreasing temperature (down to 50 °C). This artisanal technique gives rise to a selection of a microbiota derived from raw milk's microbiota that survives the various steps of halloumi cheese production. At the same time,

the pH of SCW becomes lower (down to a value of 3.5) as it becomes more acidic. The stream is then collected by trucks and transferred to other establishments to be used mainly as pig feed.

Worldwide, various strategies have been adopted attempting to manage SCW due to the restrictions and the demanding economic costs of its disposal. Although not so widely investigated as cheese whey [3,4], a literature review related to SCW revealed it is mainly exploited as a fermentation medium for the sustainable generation of added-value products, including biofuels [5], eco-friendly bioplastics (e.g., polyhydroxyalkanoates) [6,7] and polysaccharides, as well as for biomass production [8,9]. In addition, SCW seems to offer a promising alternative source for the development of innovative food products such as probiotic drinks [5] and vinegar [6]. On the other hand, the prospect of reducing food processing cost via the reuse of SCW as culture medium for the growth of lactic acid bacteria (LAB), a freeze-drying protective agent or as an encapsulating agent for the spray-drying of these microorganisms remains largely non-investigated, which is in contrast to previously developed procedures for cheese whey [10].

Whey streams are rapidly degraded due to their high microbial growth and the high content of nutrients, which supports the growth of the former. However, the exploitation of indigenous microbial communities as natural whey starter cultures is anticipated to display adequate adaptability and enzymatic activities for promoting the development of standardized cheese making processes as well as the safety and quality attributes of the final products. Importantly, the indigenous microbiota are usually the only ones allowed for PDO cheese production since, in this way, the product remains connected with its geographical origin. For example, this is the case for buffalo mozzarella cheese, an Italian product that gained a PDO recognition in 1996 [11], and Argentinian hard cheeses (i.e., reggianito, sardo, barra, provolone) [12]. However, because of their seasonal variation in microbial load and composition, the greatest disadvantage associated with natural whey starters is the inconstancy of their technological performances. One of the most important requirements is preparing microbial starters that are made up of selected and fully characterized indigenous strains (single or mixed). However, there are a lack of studies concerning the characterization of the endogenous LAB in halloumi SCW.

Starter culture production in an easy to use form that can guarantee the long-term delivery of viability and stability in the technological performances of the isolates is highly desirable. Starter cultures are nowadays increasingly used in concentrated forms for direct inoculation into the food matrix. The most usual form is that of dried cultures due to the low costs of storage and transport [13]. Freeze-drying (FD) and spray-drying technologies can be used for LAB preservation, although FD is more frequently employed. However, most LAB strains are sensitive to the drying process, although this was proved to be strain dependent. Changes in (a) the physical state of membrane lipids and/or (b) the structures of sensitive proteins are correlated with a loss of viability [14]. The addition of lyoprotectants such as sugars and proteins before FD is a common approach in order to improve survival and enhance the subsequent storage stability of LAB [15–17]. Among other lyoprotectant agents, whey protein hydrolysate has also been mentioned as an agent that could protect the cells of probiotics during FD and storage [18]. However, since freezing is the first step of the FD process, cryoprotection is also important. Glycerol, the most well-known cryoprotectant agent, cannot be used for FD due to its viscosity, which could lead to a sticky final dried product [19]. Therefore, it is very challenging to find an agent that could protect microbial cells during the entire FD process. Whey-based products have also been reported as cryoprotectants for improving the survival rates of LAB after FD, which suggests an additional application for this material that could lower production costs [10].

The present study aims, for the first time, to (a) isolate, identify and characterize the predominant cultivable indigenous LAB species from the SCW storage silos following the manufacture of halloumi and anari and (b) reuse SCW as a dual-used medium in a simplified one-step freeze-drying process involving growing and drying the selected isolates in SCW. In addition, the evolution of viability and metabolic activities of the

isolates in freeze-dried powder were monitored for 90 days at 4 °C to further support the valorisation approach.

2. Materials and Methods

2.1. Sample Collection and Chemical Characterization

Samples were collected by a halloumi cheese production line from the dairy industry located at Limassol, Cyprus. Briefly, raw cow's (70%), goat's (20%) and sheep's (10%) milks were pasteurized (72 °C, 15 s) and coagulated with rennet addition (33 °C, 40 min). The resulting curd was cut and further processed to halloumi, whereas the recovered whey was collected for the production of anari. Specifically, the whey was gradually heated to over 90 °C until the crumbly curds of 'anari' rose on the surface of the whey. Samples were aseptically collected (a) immediately after the production of anari (SCW0) and (b) after a 24 h incubation where the temperature was decreased down to 50 °C in a silo (SCW24). Sampling was from three independent processes during different days. The samples were used directly for analysis.

The pH of the samples was measured using a Mettler–Toledo pH Meter MP225 (Spain) equipped with a glass electrode (InLab Solids, Mettler–Toledo). The total solids (TSs) were determined according to standard methods [20], whereas total sugars and nitrogen were determined spectrophotometrically [21,22].

2.2. Enumeration and Isolation of LAB

Enumeration of LAB in the SCW0 and SCW24 samples was conducted according to ISO 11133 [23] by serial dilutions (1:10) in Maximum Recovery Diluent (MRD) (Merck, Darmstadt, Germany) and analysis by culture on MRS agar (Merck) supplemented with 0.01% cycloheximide (JK Scientific, Pforzheim, Germany) (37 °C, 72 h). Viable counts were expressed as colony-forming units (cfu). Representative isolates per each different LAB colony morphotype developed on the MRS agar were selected from the highest dilution plates (~20% of total colonies per plate) [24], purified by successive sub-culturing onto MRS agar and stored (20% glycerol, −80 °C). In total, 11 isolates were obtained; an unidentified one was also among them. The codes of the isolates are shown in Table 1.

Table 1. Identification of LAB isolates from halloumi SCW24 based on 16S rRNA gene sequencing and analysis.

Species Code	16S rRNA Gene Sequencing Data	Identity (%)
A1	*Lactobacillus delbrueckii* subsp. *lactis*	99
A2	*Lactobacillus delbrueckii* subsp. *lactis*	99
A3	*Lactobacillus delbrueckii* subsp. *lactis*	99
A4	*Lactobacillus delbrueckii* subsp. *lactis*	99
B1	*Lactobacillus delbrueckii* subsp. *jakobsenii*	99
B2	*Lactobacillus delbrueckii* subsp. *jakobsenii*	99
C1	*Lactobacillus leichmannii*	99
C2	*Lactobacillus leichmannii*	99
C3	*Lactobacillus leichmannii*	99
D1	*Lactobacillus crispatus*	99
W1	Unidentified	

2.3. Characterization of Isolates

2.3.1. Phenotypic Characterization

A Gram stain kit (Liofilchem, Abruzzo, Italy) was used to differentiate the bacteria by their cell wall properties according to the Gram staining technique. For catalase testing, a drop of 3% (*v*/*v*) H_2O_2 was added to each colony on a sterile glass slide and the generation of O_2 (bubble formation) was observed. Microscopic observation of the cellular morphology was performed with a microscope (100× magnification) (Optika Srl, Bergamo, Italy).

2.3.2. DNA Extraction and Molecular Identification

An aliquot of bacterial stocks was incubated in MRS broth at 37 °C for 24 h to obtain a sufficient amount of cell biomass. The precipitated cells obtained by centrifugation (14,000× *g* for 2 min, twice) from overnight bacterial cultures were used for DNA extraction by the GenElute Mammalian Genomic DNA Purification Kit (Sigma-Aldrich, Steinheim, Germany) according to the manufacturer's protocol.

The template for PCR assays comprised of genomic DNA from lysed cells (2 µL) and Taq PCR Master Mix (Qiagen, Valencia, CA, USA) supplemented with 1 mmol $MgCl_2$ and primers targeting the V3 region of the 16S rRNA gene (V3f, CCTACGGGAGGCAGCAG; V3r, ATTACCGCGGCTGCTGG; Eurofins Genomics, Ebersberg, Germany). The 16S rRNA (DNA) sequences of the isolates were determined as described elsewhere [25] using a Techne Progene automated DNA thermal cycler (Staffordshire, UK). The PCR products were stained with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific, Darmstadt, Germany) and examined by electrophoresis in a 1.8% (*w/v*) agarose gel with ethidium bromide under UV illumination. The PCR products were purified with Nucleo Spin PCR Clean-up Kit (Macherey-Nagel, Düren, Germany). The sequencing of the purified DNA was carried out at the Cyprus University of Technology (Limassol, Cyprus) using an ABI Prism 3130 genetic analyzer (Applied Biosystems, Foster City, CA, USA), and the bacterial species were identified by comparing the 16S rRNA sequences for each isolate with those reported in the EzBioCloud nucleotide database.

2.4. Growth in MRS Broth Modified with Lactose or Galactose

MRS–lactose and MRS–galactose broth was prepared after substitution of glucose with lactose or galactose in MRS broth and was used for testing the growth and metabolic capacity of the isolates in the presence of the specific sugar substrates. MRS–glucose was used as the control medium. The composition of the MRS media was as follows: 10 g Bacto TM peptone, 10 g beef extract, 5 g yeast extract, 1 g Tween-80, 20 g α-D-glucose (for MRS-glucose broth), 20 g β-D galactose-(1→4)-α-D-glucose (for MRS-lactose broth) or 10 g β-D-galactose (for MRS-galactose broth), 2 g trisodium citrate dihydrate, 5 g sodium acetate trihydrate, 0.1 g magnesium sulphate heptahydrate, 0.05 g manganese sulphate monohydrate, 2 g potassium monohydrogen phosphate and 1 L of distilled water [26]. The pH of the MRS media was adjusted to 6.5 with 2 M hydrogen chloride and the solution was autoclaved at 121 °C for 15 min.

All the isolates were firstly activated in MRS–glucose broth three times at 37 °C for 24 h before inoculation (2% of 107 cfu/mL) in MRS–no sugar, MRS–glucose, MRS–galactose and MRS–lactose. Microbial growth was monitored by measuring the OD_{600} value (Hitachi U-2000 spectrophotometer, Tokyo, Japan). The modified Gompertz equation given below was used to describe the growth of isolates in the MRS media [27].

$$y = A \exp\{-\exp[(\mu_m \cdot e)/A\ (\lambda_B - t) + 1]\}$$

where y is the optical density value (OD_{600}) at time t (h), A is the maximum value of optical density (OD_{600}), μ_m is the maximum specific growth rate (1/h), λ_B is the growth lag time (h) and t is the time (h).

For sugar metabolic capacity tests, extended cultivation (up to 72 h) was carried out to minimize the effect of inoculum size on the sugar utilization.

2.5. Growth in SCW

SCW0 samples were used without or after supplementation with skimmed milk (chemical composition: proteins 33%, *w/w*; lactose 54%, *w/w*; fat 1.25%, *w/w*; ash 8%, *w/w*) (SMP, Lefkonitziatis Ltd., Limasol, Cyprus) to increase the TS content up to 30% (*w/w*). The SMP was used as growth factor, source of casein micelles and lactose. The media were autoclaved at 90 °C for 15min, then inoculated with 2% of 107 cfu/mL of each activated culture and incubated at 37 °C for 24 h.

2.6. Freeze-Drying and Storage of LAB

Isolates grown in the SCW0-based media and MRS broth (control cultures) were used. For freeze-drying, a lab-scale freeze-dryer (Christ Alpha 1–2 LD plus, Osterode, Germany) was used. Cultures (12 mL) were frozen at −20 °C, followed by a freezing phase (18 h) at a shelf temperature of −20 °C and chamber pressure of 0.070 mbar. The condenser temperature was set to −52 °C in order to create a big partial pressure difference between the condenser and chamber. The residual moisture content at the end of drying was 1–2% (*w/w*).

The freeze-dried samples were packed in tubular plastic pouches (PA/PE; 150 mm thickness; permeability: oxygen, 40 mL/m^2 24 h (238C/75% r.F.); N2, 8 mL/m^2 24 h (238C, 75% r.F.); water vapour, 1.8 g/m^2 24 h (238C/85% r.F.)), heat-sealed under vacuum (99.9% vacuum level) (Besser Vacuum—EOS packaging machine (Dignano, Italy)) and stored at 4 °C away from light for 90 days.

2.7. Determination of Cell Viability

Viable counts for the LAB were determined by plating serial dilutions of each 24 h culture on MRS agar with overlay and incubating for 48 h at 37 °C. Colony counting was also performed to calculate the cell survival rate after freeze-drying. The lyophilized powders of the different isolates were suspended in a sterile saline solution and gently vortexed for 10 s. Serial dilutions were prepared from the initial suspensions, cultivated on MRS agar with overlay and incubated for 48 h at 37 °C. The results were expressed as log cfu/mL or log cfu/g.

Survival levels were expressed as the quotient of cfu/mL on MRS medium before (N0) and after (Nf) freeze-drying (and storage where appropriate), according to the following equation:

$$\text{Survival (\%)} = \text{Nf/N0} \times 100$$

2.8. Lactose, Galactose, Glucose and Lactic Acid Determination

Cell-free supernatants of MRS–lactose, MRS–galactose and MRS–glucose cultures were obtained by centrifugation at 10,000× *g* and 4 °C for 10 min. The supernatants were filtered through 0.20 μm PTFE filter (Waters, Milford, MA, USA). Glucose, galactose, lactose and lactic acid were separated on a hydrogen form cation exchange resin-based column Agilent HI-plex H (Agilent Technologies, Santa Clara, CA, USA) by isocratic elution. The isocratic system was a 5 mM sulphuric acid solution at a flow rate of 0.5 mL/min. The column was equilibrated in an oven at 65 °C. The injection volume was 10 μL. The samples were properly diluted in the mobile phase and filtered through 0.45 mm-pore-size regenerated cellulose membrane filters (Schleicher & Schuell, Dassel, Germany) before injection. The HPLC system was composed of an LC-10Advp pump (Shimadzu, Kyoto, Japan) and a refractive index detector (RID-6A, Shimadzu). Clarity software version 2.6.6 (DataApex, Prague, Czech Republic) was used for data processing. Quantification of content (g/L) was accomplished with the aid of standard curves and calculated by linear regression analysis.

2.9. Statistical Analysis

All experiments were carried out in triplicate. Statistical comparison of the mean values was performed by one-way ANOVA followed by the multiple Duncan test ($p < 0.05$ confidence level) using SPSS Statistics 24.0 (IBM, Armonk, NY, USA).

3. Results and Discussion

3.1. Microbial Counts in SCW

The SCW0 and SCW24 samples were subjected to enumeration of the predominant indigenous LAB colonies on the selective medium MRS agar. A high population of LAB was counted in SCW24 samples (6.7 ± 0.4 log cfu/mL), whereas no counts were detected in SCW0 samples. This finding can be attributed to the fact that the SCW0 samples underwent thermal treatment (85–90 °C) for 20–30 min until the crumbly curds of 'anari' (mostly

made of denatured whey protein and fat) rose to the surface of the whey [28]. However, stressed and injured cells may stay viable but nonculturable, suggesting sublethal damage; thus, after being under suitable conditions in the SCW24 samples, the cells can undergo recovery [29–31]. Microbial growth was accompanied by a reduction in the pH value, from 6.20 ± 0.09 for SCW0 samples to 4.75 ± 0.18 for SCW24 samples. This significant decrease in the pH value indicated the strong metabolic activity of the viable cells detected in SCW24 samples. The determination of bacterial species, other than LAB, that could have possibly survived the various steps of halloumi and anari cheese production and thus persist in whey (e.g., Bacillus species) [32] was beyond the scope of our research, mainly because these bacterial species originate mostly from poor hygiene conditions [33]. Our focus was on the identification and selection of LAB strains with potentially good starter culture properties.

3.2. Phenotype Characterisation and Molecular Identification of LAB

In this study, a representative number of the predominant LAB colonies (11) were isolated from halloumi SCW24 samples grown on MRS agar and then subjected to microscopic examination and phenotypic characterization for their presumptive identification and genera differentiation based on colony morphology, cell morphology, Gram's reaction and catalase test. Macroscopic evaluation of the LAB colonies showed the same morphology, appearing as small, round and white colonies of 2–4 mm. The 11 isolates were found to be Gram-positive and catalase-negative rod-shaped moderately thick bacilli with medium length and curved ends. All the above characteristics are shared by LAB [34].

The genomic DNA of each bacterial stock in glycerol was extracted after overnight incubation of the cultures in MRS agar. PCR products were prepared for identification by their 16S rRNA gene sequences and identified by comparing them with those available in the EzBioCloud database [25,35]. The application of PCR analysis showed a successful identification (99%) for all the isolates after comparison of their 16S rRNA sequences with the EzBioCloud database. The results are presented in Table 1. In particular, the analysis revealed that four different species of Lactobacillus genera constituted the dominant lactic microflora of halloumi SCW24 samples: *Lactobacillus delbrueckii* subsp. *lactis*, *Lactobacillus delbrueckii* subsp. *jakobsenii*, *Lactobacillus leichmannii* and *Lactobacillus crispatus* species, with a sequence similarity of 99% for each isolate compared with the Gene Bank sequences.

L. delbrueckii subsp. *lactis* is an industrially important lactic-acid-producing bacterium as it is widely used for the production of hard cheeses, such as Grana, Emmenthal and Provolone [36]. *L. delbrueckii* subsp. *jakobsenii* isolation is a finding that has not been reported earlier for milk-based sources. The subsp. *jakobsenii* has been previously isolated from malted sorghum wort that is used for the production of an alcoholic beverage (dolo) [37]. *L. leichmannii* is used in the production of the kumiss beverage by the fermentation of raw mare's milk and other fermented products [38,39]. Interestingly, *L. crispatus* is widely used in the manufacturing process of probiotic and dairy products [40]. The antimicrobial activities of various *L. crispatus* strains against foodborne pathogenic and spoilage species as well as against uropathogens have also been reported [41]. The above bacterial isolates were identified for the first time in halloumi SCW.

3.3. Effect of Lactose and Galactose on the Growth of SCW Isolates

Knowledge about the galactose and lactose utilization capacity of the isolates is critical in order to find certain species- or strain-specific characteristics. In this study, to understand the capacity of the isolates to grow on galactose or lactose, the isolates were cultivated in MRS–galactose broth or MRS–lactose broth, respectively. MRS containing glucose as the sole carbon source was used as a control. As shown in Table 2, when the isolates were inoculated in modified MRS–galactose or MRS–lactose for 24 h, their growth was indicated by an increase in the OD value. If the isolate did not grow within 24 h of incubation in the broth, it was discarded. This was the case only for the *L. delbrueckii* subsp. *lactis* isolates A2 and A3, which were not able to grow either in the control medium (MRS-glucose) or

in MRS–galactose and MRS–lactose. Regarding A1 of the same subspecies, the levels of growth for this isolate did not differ significantly in glucose, galactose or lactose ($p > 0.050$), reaching OD values up to 1.1. However, the OD of *L. delbrueckii* subsp. *lactis* isolate A4 in lactose was considerably higher than that obtained in galactose and comparable with that in glucose. Similarly, the growth of *L. delbueckii* subsp. *Jakobsenii* isolate B1 and B2 in MRS–lactose was as efficient as growth in MRS–glucose and slightly lower in MRS–galactose. This observation is valid for *L. leichmannii* isolates C1 and C2. Even though MRS–glucose and MRS–galactose were capable of sustaining the growth of the *L. leichmannii* isolate C3, inclusion of lactose significantly improved the growth performance of the culture, reaching OD values after 24 h of ~2.5. The growth performance of *L. crispatus* isolate D1 was similar to that of *L. leichmannii* isolate C3 in lactose, with growth reaching an OD of 2.4. Regarding the unidentified strain W1, its level of growth performance was similar to that of the *L. delbrueckii* subsp. *lactis* isolate A1, with growth reaching an OD of ~1 in all carbon sources (i.e., glucose, galactose and lactose).

Table 2. Optical density of the LAB isolates from halloumi SCW24 samples in modified MRS broth containing glucose (20 g/L), galactose (10 g/L) or lactose (20 g/L) as a sole carbon source.

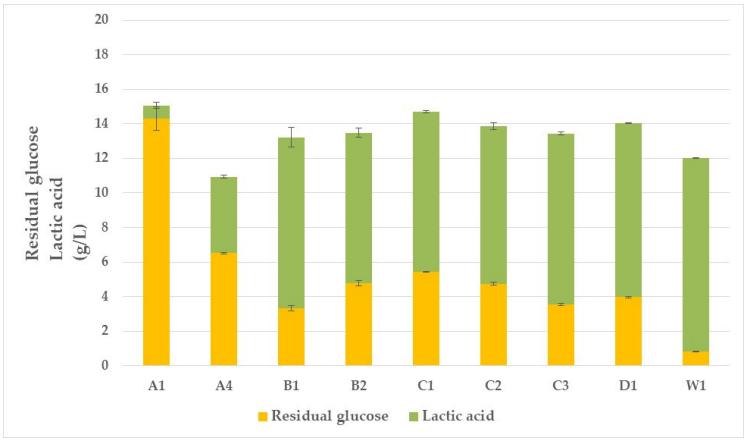
Bacterial Strain Code	16S rRNA Gene Sequencing Data	Carbon Source		
		Glucose	Galactose	Lactose
		OD_{600} [1]		
A1	*Lactobacillus delbrueckii* subsp. *lactis*	$1.047 \pm 0.634^{a,A}$	$1.1415 \pm 0.080^{a,A}$	$0.689 \pm 0.131^{a,A}$
A2	*Lactobacillus delbrueckii* subsp. *lactis*	<0.1	<0.1	<0.1
A3	*Lactobacillus delbrueckii* subsp. *lactis*	<0.1	<0.1	<0.1
A4	*Lactobacillus delbrueckii* subsp. *lactis*	$2.179 \pm 0.035^{b,B}$	$2.053 \pm 0.0090^{c,d,A}$	$2.253 \pm 0.010^{b,c,B}$
B1	*Lactobacillus delbrueckii* subsp. *jakobsenii*	$2.288 \pm 0.177^{b,B}$	$2.061 \pm 0.023^{c,d,A}$	$2.220 \pm 0.077^{b,c,B}$
B2	*Lactobacillus delbrueckii* subsp. *jakobsenii*	$2.129 \pm 0.024^{b,A,B}$	$1.794 \pm 0.489^{b,A}$	$2.223 \pm 0.052^{b,c,B}$
C1	*Lactobacillus leichmannii*	$2.310 \pm 0.014^{b,B}$	$2.069 \pm 0.004^{c,d,A}$	$2.288 \pm 0.040^{d,B}$
C2	*Lactobacillus leichmannii*	$2.158 \pm 0.013^{b,A,B}$	$2.111 \pm 0.054^{c,d,A}$	$2.176 \pm 0.042^{c,B}$
C3	*Lactobacillus leichmannii*	$2.209 \pm 0.267^{b,A}$	$2.266 \pm 0.063^{d,A}$	$2.511 \pm 0.049^{e,B}$
D1	*Lactobacillus crispatus*	$2.402 \pm 0.042^{b,B}$	$2.096 \pm 0.107^{c,d,A}$	$2.471 \pm 0.074^{e,B}$
W1	Unidentified	$1.025 \pm 0.567^{a,A}$	$1.000 \pm 0.090^{a,A}$	$1.020 \pm 0.056^{b,A}$

[1] Mean value of three independent experiments after 24 h of incubation. Different lowercase superscript letters next to mean values in the same column indicate that there are significant differences between them ($p < 0.05$). Different uppercase superscript letters next to mean values on the same line with indicate that there are significant differences between them ($p < 0.05$).

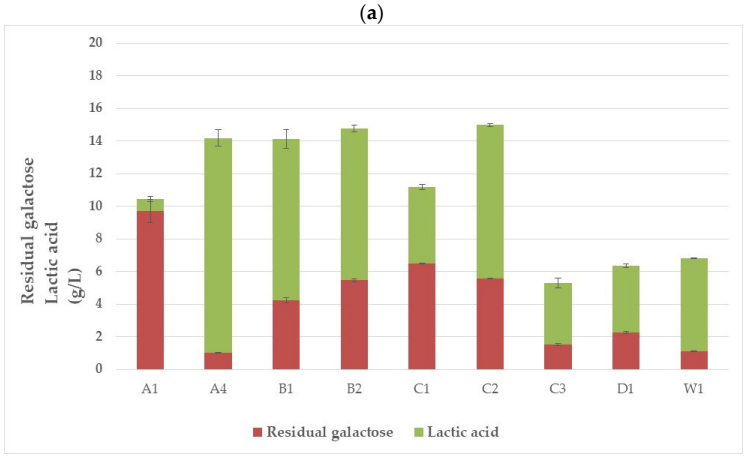
On average, the OD values of the isolates fell into the values found in the literature for various LAB strains (e.g., *L. helveticus*, *L. rhamnosis*, *L. lactis*, *L. acidophilus*, *L. casei*, and *L. sakei*) grown in MRS media without or with supplementation with various carbon sources, e.g., glucose, lactose or maltose [42–44], or in different media such as SMP [45]. From the results, it is clear that lactose and galactose are suitable carbon sources for the growth of the isolates. Given our interest in using SCW as an alternative culture medium for growing the LAB isolates, this is an important finding. Next, it was considered important to study the lactose and galactose utilization capacity of the LAB isolates.

3.4. Examination of Glucose, Galactose and Lactose Utilization Capacity of the SCW Isolates

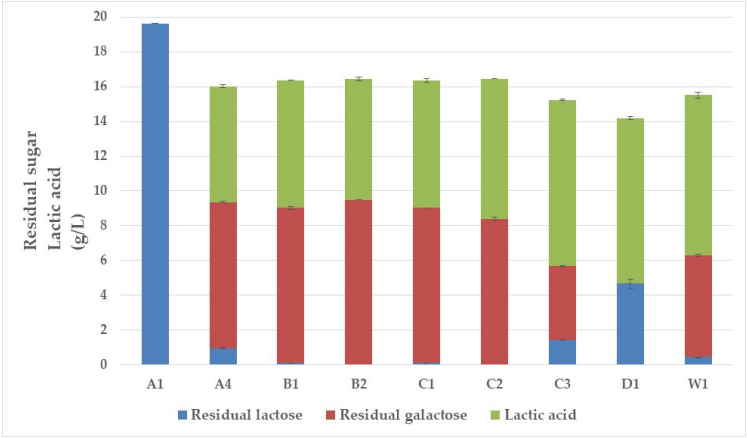
The capacity of the isolates to utilize glucose, galactose and lactose is depicted in terms of residual sugars and lactic acid production (Figure 1a–c). The *L. delbrueckii* subsp. *lactis* isolates A2 and A3, which were not able to survive in MRS-based media, were not further tested.



(**a**)



(**b**)



(**c**)

Figure 1. Glucose, galactose and lactose catabolic capacity of the isolated lactic acid bacteria from halloumi SCW24 samples and lactic acid production in different media (37 °C, 24 h): (**a**) MRS–glucose, (**b**) MRS–galactose and (**c**) MRS–lactose (A1, A4: *Lactobacillus delbrueckii* subsp. *lactis*; B1, B2: *Lactobacillus delbrueckii* subsp. *jakobsenii*; C1, C2, C3: *Lactobacillus leichmannii*; D1: *Lactobacillus crispatus*; W1: Unidentified). Error bars represent the SD of the mean.

As far as the MRS–glucose is concerned (Figure 1a), the unidentified isolate W1 produced the highest amount of lactic acid (11.2 g/L). This finding was followed by the highest amount of glucose utilization (96% of initial glucose content). *L. delbrueckii* subsp. *jakobsenii* B1, *L. leichmannii* C3 and *L. crispatus* D1 generated similar amounts of lactic acid (~10.0 g/L), resulting in marked glucose reductions of 83.3%, 82.2% and 80.0% of the initial glucose content, respectively. *L delbrueckii* subsp. *jakobsenii* B2 and *L. leichmannii* C1 and C2 produced less but still an important amount of lactic acid (8.7–9.2 g/L), with the concomitant reduction in glucose content (73–76% of the initial glucose content). *L. delbrueckii* subsp. *lactis* A1 grown in MRS–glucose could not utilize glucose efficiently (28.5% of the initial glucose content; 0.8 g/L lactic acid), which was in line with the low OD value (~1). Regarding *L. delbrueckii* subsp. *lactis* A4, although a high OD value was determined along with a reasonable amount of glucose utilized (67.5% of initial glucose content), it was observed that low amount of lactic acid was produced by this isolate (4.4 g/L).

As shown in Figure 1b, *L. delbrueckii* subsp. *lactis* A4 was more capable of using galactose (89.8% of the initial galactose content) than glucose. Noticeably, this isolate also generated significantly more lactic acid from galactose (13.2 g/L) than it did from glucose catabolism. Although a similar amount of galactose was utilized by the unidentified strain W1 (89% of initial galactose content), significantly less lactic acid was produced by this isolate (5.7 g/L), and more importantly this was lower than that from glucose. More importantly, *L. leichmannii* C3 and *L. crispatus* D1 could utilize galactose (84.7% and 77.3% of the initial galactose content, respectively) as efficiently as these isolates could utilize glucose but produced a low amount of lactic acid (3.8 g/L and 4.1 g/L, respectively). This appears to be the case for facultative heterofermentative LAB, which under glucose limiting conditions ferment hexoses to ethanol and acids (i.e., lactic acid, acetic acid and formic acid), whereas in the case of the presence of glucose in the substate ferment glucose to lactic acid [46]. However, a reverse trend was followed for *L. delbrueckii* subsp. *jakobsenii* B1, *L. delbrueckii* subsp. *jakobsenii* B2 and *L. leichmannii* C2, which produced more lactic acid (9.9 g/L, 9.3 g/L and 9.4 g/L, respectively) but catabolized less galactose (44–57%, *w/w*). Similar to the growth in MRS–glucose (Figure 1a), *L. delbrueckii* subsp. *lactis* A1 exhibited again the lowest galactose consumption as well as the lowest lactic acid production (0.8 g/L) (Figure 1b). The isolates that showed good growth in MRS–galactose medium use either the Leloir pathway (via which galactose is isomerized to glucose) or drifted to glycolysis through the tagatose-6P pathway [47–50]. High heterogeneity has also been observed by Iskandar and collaborators [51]. More specifically, they have demonstrated that bacteria of the same genus could follow the Leloir pathway, the tagatose-6P pathway, the Leloir pathway in combination with the tagatose-6P pathway or even none of these two pathways.

All the tested strains were lactose-utilization positive (Figure 1c). The W1, *L. delbrueckii* subsp. *lactis* A4, *L. delbrueckii* subsp. *jakobsenii* and *L. leichmannii* strains utilized high amounts of lactose (>93%, *w/w*) in MRS–lactose. More interestingly, lactose was exhausted (>99.5%, *w/w*) by the *L. delbrueckii* subsp. *jakobsenii* B1 and B2 and *L. leichmannii* C1 and C2 strains. However, galactose was found to be accumulated at levels (4.2–9.5 g/L) higher than those when these strains were grown in MRS–galactose (1.0–6.5 g/L). This might be due to galactose that is excreted into the growth medium and catabolized only after the depletion of lactose [52]. The level of lactose utilization was significantly lower for *L. crispatus* D1 (i.e., 76.6%, *w/w*); however, this was without any galactose accumulation (Figure 1b). In line with previous findings, *L. delbrueckii* subsp. *lactis* A1 grown in MRS–lactose could not utilize lactose efficiently (2.0% of initial lactose content), and therefore, lactic acid was not produced. Regarding lactic acid production by the rest of the isolates, it was found to lower compared with that when the strains were grown in MRS–glucose or MRS–galactose (Figure 1a–c). *L. leichmannii* C3 and *L. crispatus* D1 produced the highest noted values (9.6 and 9.5 g/L, respectively), although their lactose catabolic capacities were different (Figure 1c). Longer incubation of the isolates for 72 h revealed the capacity of *L. crispatus* D1 to completely assimilate lactose and galactose (even from 48 h), with the concomitant

production of a high amount of lactic acid (13.2 g/L). Considering the efficient lactose utilization by this strain and that the T6P pathway does not release galactose, it would be of high interest to study whether the T6P pathway is the major contributor for utilizing lactose and galactose by *L. crispatus* and its potential for reducing the accumulation of these sugar molecules in dairy fermented products, either individually or as a co-culture with other conventional or non-conventional dairy strains.

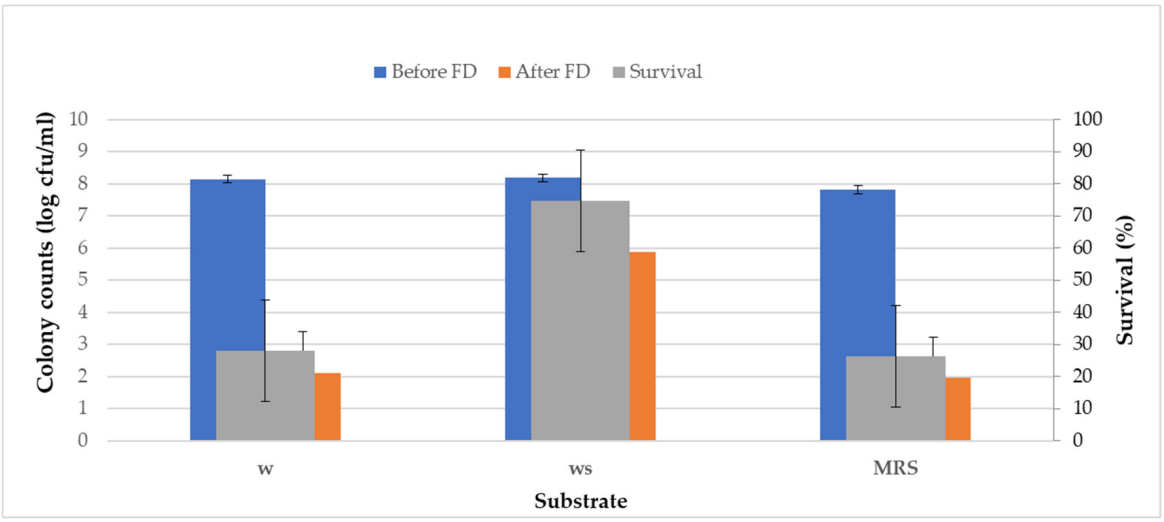
The results obtained so far demonstrate species-specific characteristics and properties for lactose and galactose catabolism. Overall, the isolated strains were able to catabolize galactose and lactose, whereas lactic acid production was comparable with the reported literature values. Reusing agro-industrial waste as low-cost culture media is one option for reducing costs, promoting environmental sustainability and reformulating bioprocesses. Thus, the next step of the current study was the examination of the SCW as a double-use medium in a simplified one-step process involving growing and freeze-drying the isolates. In particular, *L. delbrueckii* subsp. *lactis* A4, which exhibited the highest lactic acid formation with efficient galactose utilization in MRS–galactose medium; *L. delbrueckii* subsp. *jakobsenii* B1, which showed the highest lactic acid production in MRS–glucose medium; and *L. leichmannii* C3, *L. crispatus* D1 and the unidentified strain W1, which catabolized lactose with little or no galactose secretion and high lactic acid production in MRS–lactose medium, were used as the test isolates.

3.5. Effect of Freeze-Drying on the Survival of the Isolates Grown in SCW

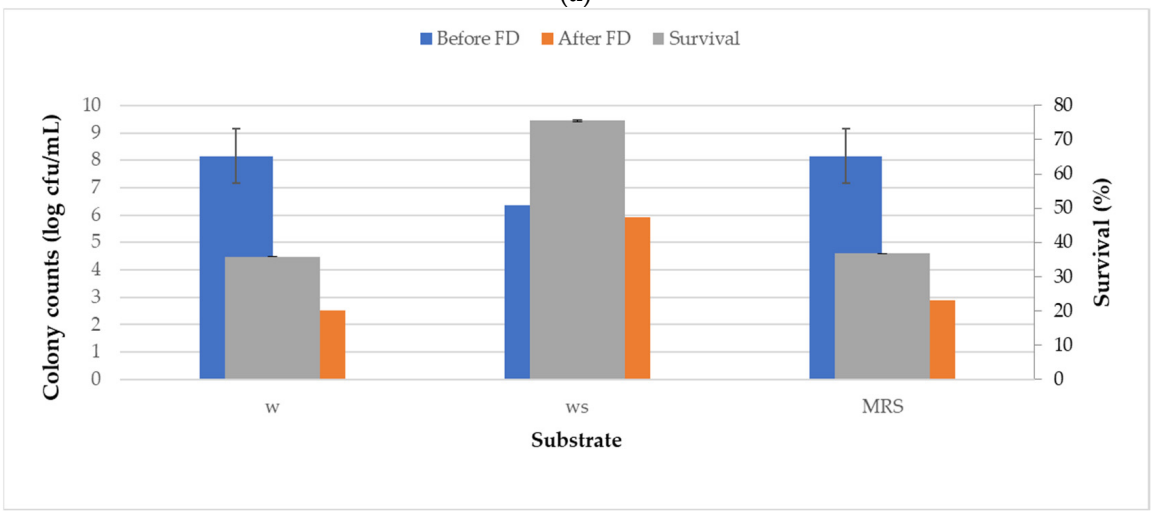
Towards the direction of valorisation of the isolates as starter cultures, the need for biomass production and their ability to withstand a freeze-drying process seemed imperative. The chemical composition of SCW0, which contains mainly lactose and minerals but also a fraction of residual fat and non-thermally precipitated nitrogen components [53], makes it an ideal medium for growing the isolates. The growth of the isolates was evaluated in SCW0 without and with SMP supplementation (TS 30% *w/v*) and in MRS–glucose medium to explore the potential differences. As shown in Figure 2, the final populations of all isolates in SCW0 did not differ significantly from those in MRS–glucose (~8 log cfu/mL) ($p < 0.05$). These results are similar to what has been mentioned before [10], considering that SCW0 has the potential to be applied as a culture medium for LAB. The SMP-supplemented medium produced similar populations of *L. delbrueckii* subsp. *lactis* A4, *L. crispatus* D1 and W1 (Figure 2a,d,e). However, the final populations of *L. delbrueckii* subsp. *jakobsenii* B1 and *L. leichmannii* C3 were negatively influenced by the SMP supplementation (~6 log cfu/mL and ~7 log cfu/mL, respectively) (Figure 2b,c). This does seem to depend on the osmotic stress in the 30% TS medium [54].

The remaining viability of the isolates after FD was expressed as the population and survival of the bacteria (Figure 2a–e). The water content of all the powders was 1–2% (*w/w*). More specifically, *L. leichmannii* C3 and *L. crispatus* D1 proved to be the most resistant after FD occurred in SCW0 (66.8% and 59.2% of the bacterial populations, respectively) (Figure 2c,d). It is interesting that the above values were found to be higher than the corresponding ones in the MRS–glucose medium (46.7% and 47.5% of the bacterial populations, respectively). In contrast, *L. delbrueckii* subsp. *lactis* A4 and *L. delbrueckii* subsp. *jakobsennii* B1 were the least resistant to FD in both SCW0 (28.1% and 35.8% of the bacterial populations, respectively) and in MRS broth (26.3% and 36.8% of the bacteria populations, respectively) (Figure 2a,b). As shown in Figure 2e, the survival of the unidentified strain W1 in powders of SCW0 was lower (47.1% of the bacterial population) than that for *L. leichmannii* C3 and *L. crispatus* D1 but higher than that for *L. delbrueckii* subsp. *lactis* A4 and *L. delbrueckii* subsp. *jakobsennii* B1. These findings agree with previous research work that highlights the fact that during freezing, drying and subsequent storage, various species of a particular genus or even different strains of the same species frequently display somewhat distinct behaviours. The membrane damage due to extracellular ice crystal formation during freezing as well as the osmotic shock and the physical state of the membrane lipids

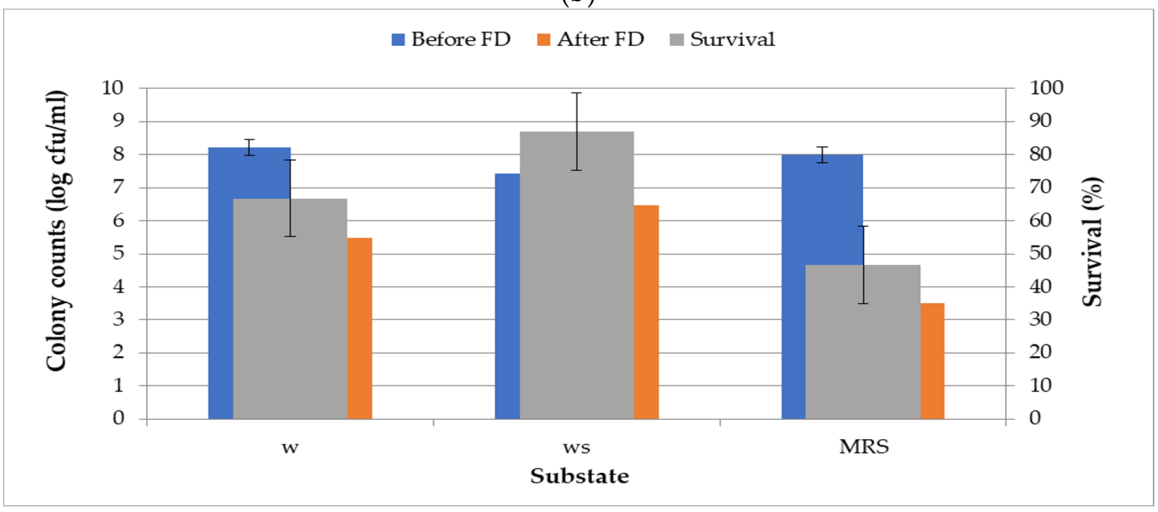
and changes in the structure of sensitive proteins are the two main causes of freeze-drying-related damage to microbial cells [55].



(**a**)

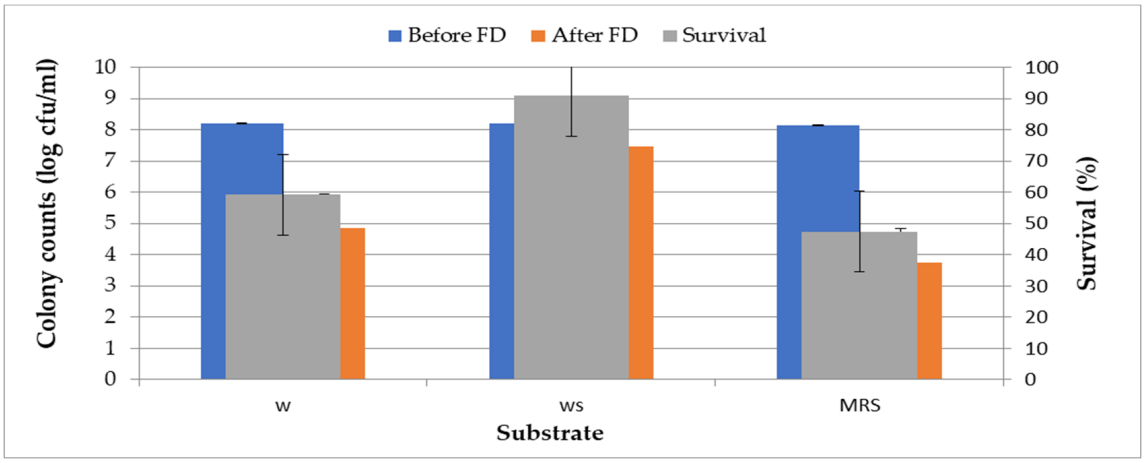


(**b**)

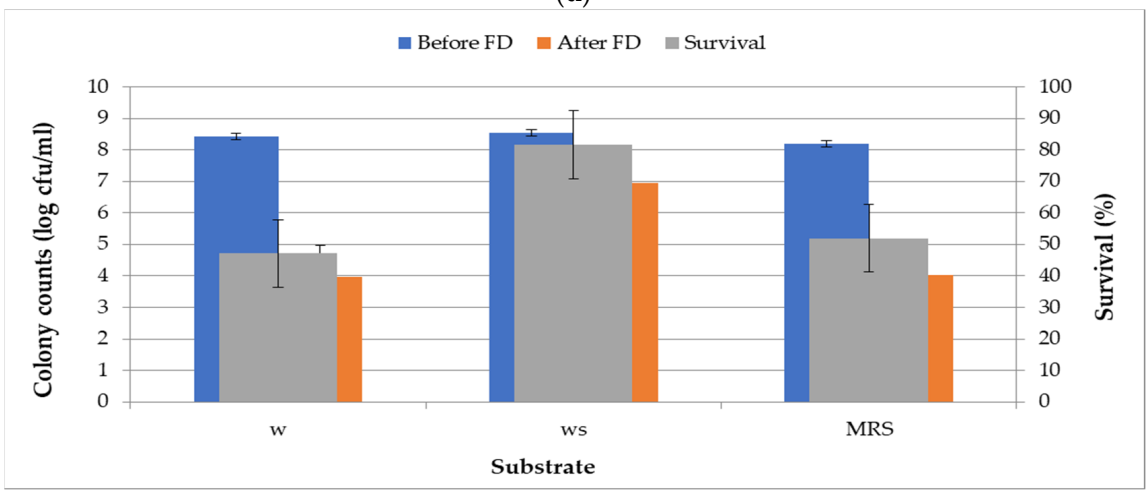


(**c**)

Figure 2. *Cont.*



(**d**)



(**e**)

Figure 2. Final bacterial populations (left Y-axis) in halloumi SCW0 without (w) and with supplementation with skimmed milk (ws) compared with MRS broth (incubation for 24 h) (MRS) and% survival (left and right Y-axis, respectively) of the (**a**) A4, (**b**) B1, (**c**) C3, (**d**) D1 and (**e**) W1 tested strains immediately after freeze-drying of the cultures.

In comparison with the powders from SCW0, the survival rates in the powders from SCW0 supplemented with SMP (30% TS) were significantly improved, though to varying degrees for each isolate. In particular, the greatest positive effect was found for *L. delbrueckii* subsp. *Lactis* A4, with an increase of approximately three-fold (74.8% of the bacterial population). There were also increases in the of survival of *L. delbrueckii* subsp. *jakobsennii* B1, *L. leichmannii* C3, *L. crispatus* D1 and the unidentified strain W1 to 2.1-, 1.3-, 1.5- and 1.7-fold, respectively (75.6%, 87.0%, 90.9% and 81.6% of the bacterial populations, respectively). Correspondingly, the populations of the isolates in the powders from the SCW0 supplemented with SMP (30% TS) were between 6 and 7.5 log units/mL (Figure 2a–e). The increased bacterial tolerance triggered by SCW0 being supplemented with SMP is consistent with research showing that FD of LAB using dairy-based protein sources and different sugars as protective agents (e.g., SMP, lactose, sucrose, whey protein concentrate, caseins and their combinations) offers significant protection from the stressful conditions employed in this process [6]. SMP proteins serve as a protective coating on the cell wall, extending bacterial tolerance against freezing and drying [56]. Additionally, SMP consists of a number of solutes such as phosphates and citrate that may protect cells from the damage caused by freezing and drying by regulating pH and promoting the stability of the cell membrane components. Alternatively, it could be because high osmolality during

growth may create enough time to accumulate the compatible intracellular solutes, either by synthesis per se or by uptake from the environment, to adapt to the adverse environments or trigger the stress response [54].

3.6. Survival of Freeze-Dried SCW Isolates after Storage

The examination of the feasibility of long-term storage of the isolates was the next crucial step of this study. Specifically, vacuum packaged freeze-dried A4, B1, C3 and D1 isolates were subjected to storage at 4 °C for three months. W1 was also studied but its survival rates were lower than 54% after 30 days, so it was not further examined. In Figure 3, the survival rates of the examined strains during storage for 30, 60 and 90 days are illustrated. High survival rates for *L. delbrueckii* subsp. *lactis* A4 were found (≥99.9%) during the three-month storage period. *L. delbrueckii* subsp. *jakobsenii* B1 was the next most resistant strain, exhibiting survival rates from 82.6% after 90 days up to 96.4% after the first 30 days of storage. A similar pattern was observed for *L. leichmannii* C3 (survival rates of 87.5%, 81,2% and 79.0% after 30, 60 and 90 days of storage). *L. crispatus* D1 displayed the lowest survival rates, i.e., 75.1% after the first 30 days, whereas after 60 and 90 days similar survival rates were found (64.3% and 63.5%, respectively). Thus, the survival rate is strain dependent, as already reported for LAB and other bacterial strains [55,57,58]. Moreover, after the 90-day storage period, the capacity of the isolates to utilize lactose and to produce lactic acid in MRS–lactose medium was found to be satisfactory.

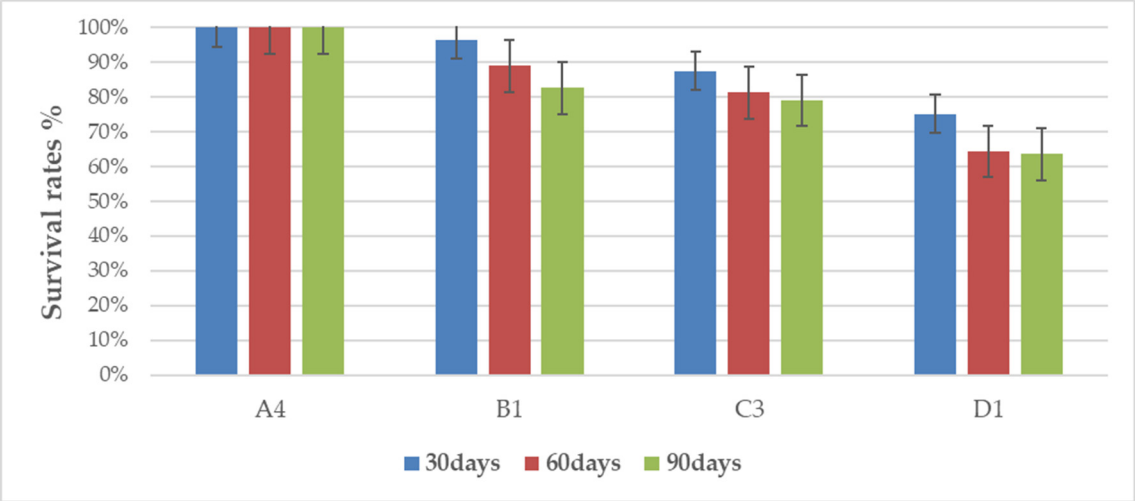


Figure 3. Survival rates of the freeze-dried strains A4, B1, C3 and D1 using SCW0–SMP during 30, 60 and 90 days of storage packed under vacuum (A4: *Lactobacillus delbrueckii* subsp. *lactis*; B1: *Lactobacillus delbrueckii* subsp. *jakobsenii*; C3: *Lactobacillus leichmannii*; D1: *Lactobacillus crispatus).* Error bars represent the SD of the mean.

4. Conclusions

Lactobacillus delbrueckii subsp. *lactis*, *Lactobacillus delbrueckii* subsp. *jakobsenii*, *Lactobacillus leichmannii* and *Lactobacillus crispatus* were isolated and identified as the dominant indigenous LAB in halloumi SCW. Lactose/galactose utilization tests demonstrated species-specific differences in galactose and lactose catabolism. Additionally, following the principles of circular economy, re-utilization of SCW as a dual medium for the growth and subsequent drying of LAB was found to be effective. Under this concept, SCW supplemented with SMP was further proved to be an efficient medium for protecting the newly isolated LAB during the freeze-drying process and long-term storage in vacuum packaging, performing efficient viability and metabolic activities. Thus, the findings of the present study add value to the halloumi SCW and, under simple directions, the dairy industry will be able to efficiently produce lactic acid bacteria in dry form for further use in other fermented dairy food products. Aiming for the current data to come into industrial

practice and to enhance the sustainable bioeconomy along with the competitiveness at both local and regional levels, the scaling-up feasibility of the proposed procedure should be investigated. Last but not least, examination of the food safety characteristics (e.g., resistance to antibiotics and production of toxins) of the newly isolated strains should also be conducted.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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