Original article

Sweet whey cheese matrices inoculated with the probiotic strain *Lactobacillus paracasei* LAFTI® L26

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Abstract – Consumption of dairy products containing viable probiotic strains has increased dramatically in recent years, owing to general health claims associated therewith. This trend has boosted diversification of the portfolio of said products, including whey cheese matrices. However, taking into account the relatively poor organoleptic and textural features of these matrices, improvement is in order via incorporation of selected additives, provided that viability of the strains is duly assayed. *Lactobacillus paracasei* LAFTI® L26 was accordingly incorporated into whey protein solid matrices, in the presence of several additives aimed at enhancing their organoleptic appeal and textural performance. These matrices were produced from a combination of either ovine or bovine whey (or a mixture thereof) with ovine milk, and were inoculated at 10% (v/v) with the probiotic strain. Sugar, sugar and aloe vera, sugar and chocolate, and sugar and jam were further added, and the resulting products were then stored at 7 °C for 21 d. In general, viable cell numbers remained high in all experimental matrices throughout storage. Despite the observed low extents of breakdown, proteolytic activities by the end of storage were higher in matrices containing jam. Furthermore, *L. paracasei* partially converted lactose into lactic acid in these matrices. Additives enhanced the organoleptic features of whey cheeses, and produced different textural patterns. The higher sensory scores were attained by matrices containing sugar: sugar and aloe vera received the best scores by 3 d of storage, but these scores decreased as storage time elapsed.

probiotics / proteolysis / glycolysis / texture / novel foods / dairy foods

摘要 – 接种副干酪乳杆菌 **LAFTI® L26** 的甜乳清干酪。近年来, 随着人们健康意识的提高, 含有活益生菌的乳制品消费量急剧增加, 由此推动了活菌乳制品的多样化, 如乳清干酪基料等。由于这类制品的感官和质构都相对较差, 需要添加合适的添加剂和菌株对其加以改进。为了改善乳清干酪的感官和质构特性, 将副干酪乳杆菌 LAFTI® L26 接种到含有多种添加物的乳清蛋白基料中。以 10% (v/v) 接种量将为副干酪乳杆菌接种到由绵羊乳清或牛乳清 (或二者的混合物) 与绵羊奶的混合物中, 然后分别添加糖、糖和库拉索芦荟、糖和巧克力、糖和果酱, 将最终产品在 7 °C 下贮藏 21 d。在整个贮藏期内所有样品的活菌数均较高。尽管含有果酱的样品在贮藏末期出现较低程度的断裂, 但是蛋白水解活力较高。此外, 在这些混合基料中副干酪乳杆菌还能将部分乳糖转化成乳酸。添加剂改善了乳清干酪的感官, 并产生了不同的质构模式。添加糖、糖和芦荟的乳清干酪在贮藏 3 天时的感官评分较高, 但随着贮藏时间的延长, 感官分值下降。

益生菌 / 蛋白水解 / 糖酵解 / 质构 / 新食品 / 乳制品

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Résumé – Matrices fromagères de lactosérum doux inoculées avec la souche probiotique *Lactobacillus paracasei* LAFTI® L26. La consommation de produits laitiers contenant des souches de probiotiques viables a beaucoup augmenté ces dernières années, en raison des allégations de santé qui leur sont attribuées. Cette tendance a favorisé la diversification de cette gamme de produits, incluant les matrices fromagères de lactosérum. Cependant, compte tenu des propriétés organoleptiques et texturales relativement pauvres de ces matrices, leur amélioration est recherchée par l'ajout d'additifs sélectionnés, sous réserve que la viabilité des souches soit dûment testée. *Lactobacillus paracasei* LAFTI® L26 a donc été incorporée dans des matrices solides de protéines de lactosérum, en présence de plusieurs additifs destinés à améliorer leur qualité organoleptique et leur texture. Ces matrices étaient produites à partir d'un mélange de lactosérum soit ovin soit bovin (ou un mélange des deux) et de lait de brebis, et étaient inoculées à 10 % (v/v) avec une souche probiotique. On ajoutait ensuite du sucre, du sucre et de l'aloe vera, du sucre et du chocolat, ou du sucre et de la confiture ; les produits obtenus étaient alors stockés à 7 ˚C pendant 21 jours. En général, le nombre de cellules viables restait élevé dans toutes les matrices expérimentales et tout au long du stockage. Malgré le peu de dégradation observée, les activités protéolytiques étaient plus élevées en fin de stockage dans les matrices contenant de la confiture. De plus, *L. paracasei* convertissait partiellement le lactose en acide lactique dans ces matrices. Les additifs amélioraient les propriétés organoleptiques des fromages de lactosérum et produisaient différentes sortes de texture. Les scores sensoriels les plus élevés étaient obtenus par les matrices contenant du sucre ; les matrices contenant sucre et aloe vera avaient cependant les meilleurs scores à 3 jours de stockage, mais ne les conservaient pas au cours du temps.

probiotique / protéolyse / glycolyse / texture / aliment nouveau / produit laitier

1. INTRODUCTION

Several distinct dairy matrices have to date been tested as carriers of probiotic bacteria, which include infant instant milk formulas and powdered milk, as well as fermented milk products, e.g. yoghurt [31], ice cream [15], dairy desserts [23] and cheese [11, 14]. Cheese may offer a number of advantages over yoghurt-type products, especially in terms of delivery of viable probiotics, because of its higher pH, fat content and mechanical consistency. All of these features provide extra protection to probiotic strains, as they are particularly susceptible to the conditions prevailing during storage prior to ingestion (and throughout the gastrointestinal tract) [34]. Cheese has accordingly been the focus of dedicated research efforts, which encompass a number of varieties: white brined cheese [10], Cheddar cheese [6, 7, 11, 32], Gouda cheese [14], cottage cheese [3, 30], Crescenza cheese [12], semi-hard goat's cheese [13], Argentinean fresco cheese [36], Turkish white cheese [19] and Brazilian Minas fresh cheese [4].

Requeijão is a soft cheese obtained from whey proteins upon thermal precipitation; this traditional Portuguese dairy product resembles Ricotta cheese. Whey proteins possess important features pertaining to human nutrition and health, which easily override their classical low added value. Additionally, antimicrobial and antiviral actions, immune system stimulation, and anti-carcinogenic activity have been claimed for such whey proteins as α-lactalbumin, β-lactoglobulin, lactoferrin, lactoperoxidase, serum albumin and glycomacropeptide [25]. All of these are present, in concentrated form, in whey cheese.

Based on the above pieces of evidence, incorporation of probiotic bacteria (e.g. strains of *Lactobacillus acidophilus*, *Lactobacillus paracasei*, *Lactobacillus brevis*, *Bifidobacterium lactis* and *Bifidobacterium animalis*) was successfully attempted in whey cheese matrices [24]: all strains tested were able to maintain a high viability (above 10^7 cfu·g^{-1}) for 28 d of storage under refrigeration. However, suitable criteria for technological selection of probiotic strains encompass not only an intrinsic

ability to maintain high viable populations (in the typical range 10^6–10^8 cfu·g^{-1}), but also a set of appealing sensory features [20]. To meet with commercial success, a food product should in fact exhibit both types of properties. *Lactobacillus paracasei* LAFTI® L26 was thus used not only because of its good viability in the matrices at stake, but also for its probiotic features, e.g. survival in the human gastrointestinal tract [37] and an antimicrobial effect against *Escherichia coli* [29].

In general, actively metabolising microorganisms in dairy matrices play roles in glycolysis (i.e. lactose consumption, and concomitant organic acid production), proteolysis (i.e. protein breakdown, and concomitant peptide and amino acid release) and lipolysis (i.e. triglyceride breakdown, and concomitant free fatty acid release). These bacterium-mediated activities contribute differently (but somehow complementarily) to the final organoleptic profiles of said dairy products, either favourably, or via generation of off-flavours. Incorporation of certain food additives may modulate said metabolic pathways, as well as reduce (or even avoid) undesirable tastes.

Therefore, the aim of the present research effort was to assess the effect of incorporating a number of additives into probiotic whey cheese matrices, upon the metabolic profile of *L. paracasei*, and upon textural and sensory characteristics of those matrices stored under refrigeration.

2. MATERIALS AND METHODS

2.1. Microorganism source

Lactobacillus paracasei LAFTI® L26 was obtained as a DELVO-PRO® freeze-dried, concentrated starter culture from DSM (Moorebank, Australia). In order to prepare an inoculum adequate for whey cheese matrices, the bacteria were first grown in MRS broth (Merck, Damstadt, Germany), and thereafter cultured twice in skim milk (Oxoid, Hampshire, UK); in both cases, incubation was at 37 °C for 24 h.

2.2. Whey cheese manufacture

Experimental manufacture of whey cheeses was carried out using whey released as a by-product of full-fat cheese-making, from a mixture of 90% (v/v) ovine and 10% (v/v) bovine raw milks, blended with raw ovine milk to 10% (v/v) – both provided by Marofa II (Figueira de Castelo Rodrigo, Portugal). Upon arrival, these liquid feedstocks were immediately refrigerated to 7 °C, and stored thereafter at that temperature for up to 2 d.

Five whey cheese replicates were manufactured from whey batches on two different days. The equipment used was a 100-L double-walled, steam-supplied vat heated with burning gas, with controls for stirring rate and temperature. Whey was poured into this vat and slowly heated, under gentle stirring, up to 87–90 °C. Once precipitation started, ovine milk was added at the aforementioned ratio under continued stirring. When the temperature reached 95 °C, it was maintained at this level for 20 min. No pH correction was necessary (as is usually done in Ricotta cheese-making), because pH was essentially that which provides the highest protein precipitation yield. The resulting floating curd was collected from the surface with a sterile skimmer, and poured into sterile plastic moulds – which were duly covered, and left to drain for 20 min, under a constant force of ca. 2 N·cm^{-2}. After draining, whey cheese matrices were left to cool to room temperature (ca. 20 °C) for an extra 30 min.

In each (duplicated) batch, the resulting curd was inoculated with the probiotic culture at 10% (v/v) – which had been previously prepared as described above; this inoculum permitted the intended initial level of ca. 10^7 cfu·g^{-1} to be attained. One of the batches was directly used as

Table I. Physicochemical composition (average ± standard deviation) of whey and milk feedstocks.

	Component			
Matrix	Fat (%, w/w)	Protein (%, w/w)	Lactose (%, w/w)	pH
Whey	0.53 ± 0.02	0.78 ± 0.03	4.90 ± 0.21	5.30 ± 0.03
Milk	6.18 ± 0.02	8.07 ± 0.01	4.35 ± 0.43	5.98 ± 0.01

control (matrix C), whereas the remaining three batches were then separately mixed with: 5% (w/w) sugar (matrix S), 5% (w/w) sugar and 3% (w/w) aloe vera (matrix AV), 5% (w/w) sugar, 4.5% (w/w) chocolate fibre and 4.7% (w/w) chocolate powder (matrix CH), and 10% (w/w) strawberry jam (matrix J). All matrices were then vigorously stirred for 5 min with an electric mixer (Kenwood, UK) with a whisk adapted to the rotating shaft, and equally distributed into sterile 100-mL flasks, that were immediately capped so as to simulate closed packages, and stored at 7 °C for up to 21 d.

Aseptic conditions were assured throughout the whole manipulation protocol, in order to prevent contamination.

2.3. Microbiological analyses

Duplicate sampling of all types of whey cheese matrices (via 8-g aliquots) took place at 0, 3, 7, 14 and 21 d. Dilution was performed with sterile 2% (w/v) aqueous sodium citrate (Merck), and homogenisation took place for 3 min at 260 rpm, in a Stomacher Lab Blender 400 (Sewer Medical, London, UK). Serial decimal dilutions were later prepared with 0.1% (w/v) peptone water (Sigma, St. Louis, MO, USA), and plated in duplicate on several selected media: plate count agar (PCA, Merck), incubated for 2 d at 30 °C, for total viable counts of aerobic mesophilic bacteria; Rogosa agar (Merck) supplemented with acetic acid at 96% (v/v) (Sigma) to achieve pH 5.2, incubated for 3 d at 37 °C, for viable counts of *L. paracasei*; Baird Parker

Medium (Lab M), supplemented with sterile egg yolk tellurite (Lab M), incubated for 2 d at 37 °C, for viable counts of *Staphylococcus aureus*; Violet Red Bile Glucose Agar (VRBGA, Lab M), incubated for 1 d at 37 °C, for viable counts of *Escherichia coli*; Pseudomonas agar (Merck), incubated for 1 d at 37 °C, for viable counts of *Pseudomonas aeruginosa*; kanamycin aesculin agar (Merck), incubated for 1 d at 37 °C, for viable counts of *Enterococcus*; and Rose Bengal Chloramphenicol Agar (RBCA, Merck), incubated for 5 d at 25 °C, for viable counts of moulds and yeasts. All aforementioned media were plated using the classical technique [26], except in the case of VRBGA and RBCA, which followed the pour and spread-plate techniques, respectively, as described in detail elsewhere [5].

2.4. Chemical analyses

Whey and milk used for manufacture of the experimental whey cheeses, as well as the final products, were submitted to physicochemical analyses in triplicate, which included pH, fat, protein and lactose contents, using a LactoScope Advanced FTIR (Delta Instruments, The Netherlands). The results obtained for milk and whey are tabulated in Table I.

2.5. Proteolysis assessment

Duplicated samples of whey cheese taken at 0, 14 and 21 d of storage were assayed for proteolysis: water-soluble nitrogen (WSN), as well as nitrogen soluble

in 12% (w/v) trichloroacetic acid (TCA-SN) and in 5% (w/w) phosphotungstic acid (PTA-SN), were determined by Kjeldhal, as described elsewhere [21,33], except that a Stomacher Lab Blender was used for homogenisation, and that the supernatant obtained was filtered through Whatman No. 42 filter paper. Proteolysis indices, i.e. WSN-TN %, TCA-TN % and PTA-TN %, were then calculated as the percent ratios of WSN, TCA-SN and PTA-SN, respectively, to total nitrogen (TN).

2.6. Glycolysis assessment

Duplicated samples of whey cheese, taken at 0, 7 and 21 d of storage, were assayed for glycolysis: organic acids and sugars were quantified by HPLC in a single run, based on calibration curves previously prepared with appropriate chromatographic standards, using an apparatus from Merck LaChrom (Fullerton, CA, USA), with an Aminex HPX-87X cation exchange column from BioRad (Richmond, CA, USA). The flow rate was 0.5 mL·min^{-1}; 0.0025 mol·L^{-1} H$_2$SO$_4$ (Merck) was used as eluant, and detection was by refractive index at 60 °C for sugars, and UV absorbance at 220 nm for organic acids. Prior to analysis, all samples were pretreated as follows: 4 g of sample was homogenised with 30 mL of 0.5 mol·L^{-1} aqueous perchloric acid (Merck), for 3 min in a Stomacher Lab Blender 400, allowed to stand for 2 h at a refrigeration temperature in a closed vessel, and filtered through a 0.22-μm membrane Syrfil filter (Nucleopore, Cambridge, MA, USA).

2.7. Textural analyses

Replicated samples of whey cheese, taken at 0, 3, 7, 14 and 21 d of storage, were assayed via measurement of the force-time curve with a TA.XT apparatus (Stable Micro Systems, Surrey, UK).

A 5-kg load cell was calibrated with a 2-kg weight. The probe used was P/30c (a 30' conical device, made of perspex), and tests were performed directly in the flasks (in triplicate), at three different locations on the sample. The samples were identical, in terms of weight and shape. A typical "mastication test" profile was used, which precludes two consecutive compressions at controlled room temperature (25 °C). The compression distance used was 20 mm, to ensure that the sample did not fracture before the second compression. The two consecutive compressions were performed automatically, at a test speed of 5 mm·s^{-1}. This test permitted one to measure five attributes: hardness, cohesiveness, gumminess, adhesiveness and springiness. To measure softness, a single cycle of compression was used at a test speed of 2 mm·s^{-1} and a compression distance of 20 mm. The inverse value of the maximum peak force, during the compression applied to the sample, defines softness.

Hardness is defined as the maximum peak force during the first compression cycle (first bite) – a concept that has often been substituted by the term firmness.

Gumminess is the product of hardness by cohesiveness. It is a characteristic of semisolid foods, with a low degree of hardness and a high degree of cohesiveness.

Cohesiveness is defined as the ratio of the positive force area during the second compression cycle, to that during the first one. Cohesiveness is thus a measurement of the rate at which the material disintegrates, under mechanical action, and tensile strength is a manifestation thereof.

Adhesiveness is the negative force area for the first bite, and represents the work required to overcome the attractive forces between the surface of a food and the surface of other materials with which the food comes into contact (measured as the total force necessary to pull the compression plunger away from the sample).

Finally, springiness (originally called elasticity) is related to the height that the food recovers, during the time elapsed between the end of the first bite and the start of the second bite.

2.8. Sensory analyses

A sensory panel trained for acceptance scoring assessed the coded matrices in random order. The panel consisted of 15 members, who underwent previous training with sensory analyses of dairy products, with ages ranging from 25 to 45 years old. Sample pieces were placed into airtight plastic containers, and were conditioned at room temperature for 15 min before evaluation, so as to guarantee that samples were consumed still fresh. Duplicated samples were evaluated at room temperature (20 °C), but only upon previous confirmation of microbiological safety, by the consumer panel using a 9-point hedonic scale (in which 1 corresponds to "very bad", and 9 to "very good"). Between analyses, each member of the panel took a glass of water and a few flavour-inert biscuits, so as to avoid carry-over tastes from the previous analysis. During classification, general remarks about aroma, consistency, flavour and acidity were also recorded by the members of the sensory panel.

2.9. Statistical analyses

Shapiro-Wilke normality tests were applied to initial chemical composition, viable cell counts, pH, acidity, glycolysis, proteolysis and textural experimental raw data, and most were found not to satisfy the homoscedasticity hypothesis. Since data transformation was not successful in all cases, a non-parametric test, e.g. Friedman's test, was eventually applied to said data. This test is used to detect differences in treatments across multiple test attempts, and is also known as two-way analyses in ranks. Since statistical differences were detected between values, the influence of storage time was assessed via a Wilcoxon test. This test involves comparison of differences between measurements, so it requires that the data be measured at an intermediate level of measurement (storage time). It does not require assumptions to be satisfied pertaining to the form of the distribution of experimental measurements, and should therefore be used whenever the distributional assumptions that underlie the *t*-test cannot be fully satisfied. Differences between the five types of whey cheeses were analysed using Mann-Whitney tests, which assess whether two samples of observations come from the same distribution. All tests were performed to a 5% significance level, using SPSS software (v. 15.0, SPSS, Chicago, IL, USA).

Tentative correlations between viable cell counts, pH, acidity and glycolysis, and also between textural parameters, were checked via Pearson's tests. This type of test was performed to a 1% significance level.

3. RESULTS AND DISCUSSION

3.1. Microbiological profiles

The viable cell numbers of the experimental inocula ranged between 10^9 and 10^{10} cfu·g^{-1}, so as to originate a whey cheese bearing a probiotic character: 10^8 cfu·g^{-1} is indeed the most commonly accepted threshold thereof [16,18]. The nature of this matrix does not lead to significant syneresis following manufacture, so no microbiological analyses of any putatively expelled effluent were warranted. Viable cell numbers in whey cheeses increased by ca. 1.5 log cycles during the 21-d storage at 7 °C, in all experimental matrices (Fig. 1). The highest increase
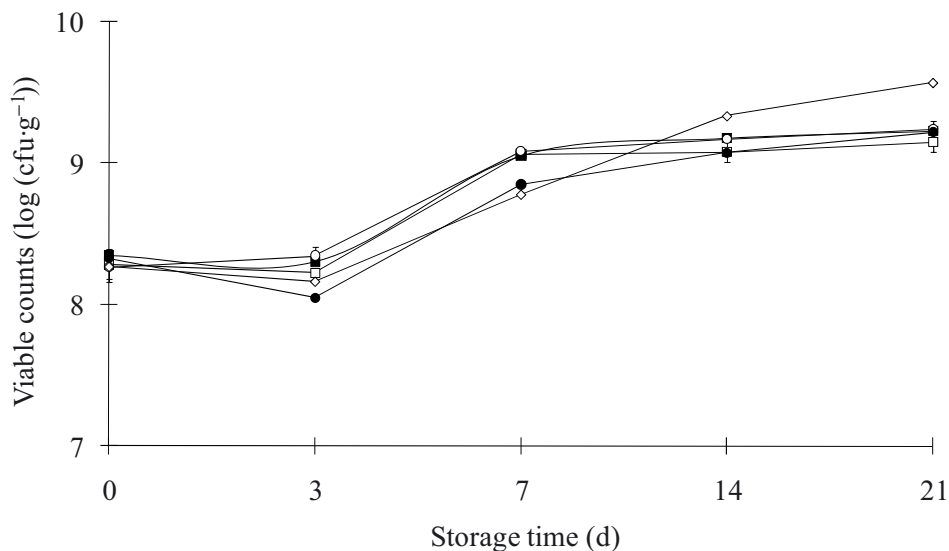
Figure 1. Evolution (average ± standard deviation), throughout storage at 7 °C, of viable counts of *Lactobacillus paracasei* in whey cheese matrices: (□) with no additives (C), (■) with added sugar (S), (○) with added sugar and aloe vera (AV), (●) with added jam (J), and (◇) with added sugar and chocolate (CH).

was perceived after only 3 d of storage: no statistically significant differences between matrices were actually perceived before this time ($P > 0.05$). A slight increase was observed by 21 d in matrix CH, but no statistical differences were found for microbiological viability among the five matrices ($P > 0.05$). Hence, the numbers of viable cells were not affected by incorporation of the additives considered.

In a previous research work [24], the strain used (which had the old designation *L. paracasei* LCS1) was reported to increase its viable cell counts when inoculated into matrices based on bovine whey and milk, and with added salt and sugar. One of the major reasons to select this strain for the present work was the good technological features exhibited so far, particularly the good viability in solid whey matrices, as well as its intrinsic probiotic properties. In particular, this strain displays a good viability profile upon incorporation

in such dairy products as Cheddar cheese [28] and yoghurt [27]. Strains of *L. paracasei* were also shown to exhibit good viability in other related dairy products, e.g. fermented milk [8,27], Argentinean Fresco cheeses [36] and Brazilian Minas fresh cheese [4].

Besides its probiotic functions and excellent growth in the whey cheese matrices, the aforementioned strain was chosen specifically because of its metabolic features, since it is known to be a facultative heterofermentative Lactobacillus (FHL). This type of added culture is known to actively interact with undesirable adventitious microbiota, by competing for the limited supply of substrates and growth factors present in cheese [22].

No external contamination was found in any experimental whey cheese throughout storage time (results not shown), at least to the limit of detection of the enumeration technique chosen (i.e. 10^3 in

Table II. Physicochemical composition (average ± standard deviation) of whey cheese matrices upon manufacture: (C) control, (S) with added sugar, (AV) with added sugar and aloe vera, (CH) with added sugar and chocolate, and (J) with added strawberry jam.

	Component				
Matrix	Fat (%, w/w)	Total protein (%, w/w)	Water content (%, w/w)	pH	Lactose (mg·g^{-1})
C	14.40 ± 0.00^a	13.21 ± 1.26^a	65.43 ± 0.98^a	5.56 ± 0.01^a	29.97 ± 1.32^a
S	11.60 ± 0.04^b	13.52 ± 0.738^a	70.85 ± 3.74^{ab}	5.49 ± 0.00^b	23.52 ± 2.14^a
AV	9.89 ± 0.07^b	99.79 ± 1.04^a	69.57 ± 0.62^{ab}	5.05 ± 0.07^c	21.89 ± 0.02^a
CH	8.20 ± 0.04^c	13.70 ± 0.775^a	66.57 ± 0.73^{ab}	5.52 ± 0.00^c	23.45 ± 2.05^a
J	10.20 ± 0.34^d	13.02 ± 2.28^a	71.11 ± 0.31^b	5.33 ± 0.00^c	24.23 ± 1.09^a

^{a,b,c} Means within the same column, marked with the same letter, do not differ from each other ($P > 0.05$).

general and 10^2 for coliforms). Moreover, no increase in total viable cell numbers was detected on PCA. Consequently, the mesophilic bacterial numbers obtained were accounted for by the viable cell numbers of only *L. paracasei.*

3.2. Physicochemical profiles

The pH values of whey and milk used for manufacture of the experimental whey cheeses, despite their low values (which are an expected consequence of the incipient microbial-mediated acidification of milk during cheesemaking), can be considered acceptable for manufacture of those matrices. Denaturation of whey proteins can in fact take place in the pH range 5.5–6.0. The fat, protein and lactose contents, as well as the pH of ovine milk added during manufacture of whey matrices (Tab. I), had accordingly typical values for this type of milk, except for pH, which was on the low side, mainly due to transportation of milk before manufacture that might have permitted some degree of microbial-driven acidification.

Differences were found between matrices in terms of physicochemical parameters – which confirms the direct effect of incorporating additives upon whey cheese matrix composition ($P < 0.05$). These differences were found in terms of fat,

water content and pH (Tab. II); matrix J possessed higher water content, certainly owing to the addition of strawberry jam, an extra source of water.

3.3. Acidification

In terms of acidification, all matrices underwent a decrease of ca. 1 pH unit (from 5.5 to 4.5) as storage time elapsed. However, acidity remained high in all five matrices, especially in the case of matrix J, and statistically significant differences between the set of matrices were found throughout storage time (Fig. 2). The acidification (and pH) were apparently not influenced by the type of matrix. It is somewhat surprising that matrix C underwent an acidification pattern similar to the others, even though it contained no added sugar. Its content of lactic acid was lower, but an apparently stronger buffering effect made its pH remain essentially the same. A higher decrease in pH, and a concomitant increase in acidity were detected when sucrose was added at higher concentrations in an oat-based drink manufactured with *Lactobacillus* [1, 35]. Matrix J underwent a higher acidification, with lower pH values and higher acidity recorded. Values of each parameter were correlated with each other, and the best correlation coefficients
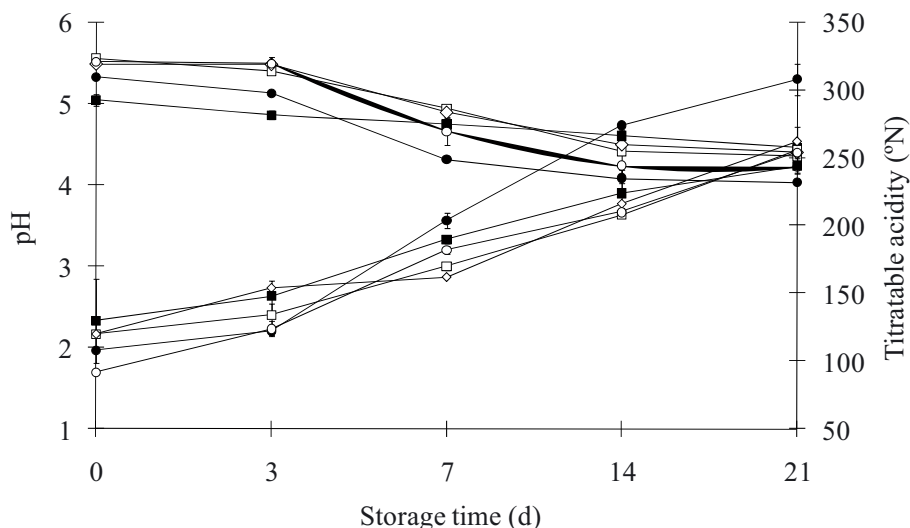
Figure 2. Evolution (average ± standard deviation), throughout storage at 7 °C, of pH and titratable acidity in whey cheese matrices: (□) with no additives (C), (■) with added sugar (S), (○) with added sugar and aloe vera (AV), (●) with added jam (J), and (◇) with added sugar and chocolate (CH).

were actually found for pH and acidification of matrix AV (r = 0.97). No statistical differences, meanwhile, were found among matrices, in terms of pH and (titratable) acidification rate ($P > 0.05$).

3.4. Proteolysis

In general, the proteolytic activity of *L. paracasei* was poor throughout storage time. Furthermore, substrate proteins were less available for hydrolysis – because they constituted a separate (solid) phase, and were also in an aggregated state (which hampered bulk access by soluble bacterial proteinases). Slight differences in proteolytic activity were nonetheless detected. For example, higher values of all nitrogen fractions were found in matrix J, by 21 d of storage. At this time of storage, statistically significant differences were found between matrices ($P < 0.05$).

The extent of proteolysis (WSN) observed (see Fig. 3a) was low. The TCA-SN

fraction contains medium and small-sized peptides, amino acids and smaller nitrogen compounds (e.g. amines, urea and ammonia). On the other hand, the PTA-SN fraction is composed of very small peptides, amino acids, and smaller nitrogen compounds other than dibasic amino acids and ammonia, which makes it a fair index of free amino acid content [2]. The ripening depth (TCA-SN) evolved in a way similar to WSN (Fig. 3b). Bacterial proteinases and peptidases are apparently able to further break down large peptides, and thus release intermediate-sized (and soluble) peptides. Nevertheless, no significant differences were found between matrices for TCA-SN ($P > 0.05$). Meanwhile, small peptides (and even free amino acids) also formed, which are soluble in PTA, and thus accounted for the high values of PTA-SN observed (Fig. 3c). In this case, differences were found especially between matrix C and the others, by 14 d of storage ($P < 0.05$). By the end of storage, matrix J exhibited the highest PTA-SN ($P < 0.05$).
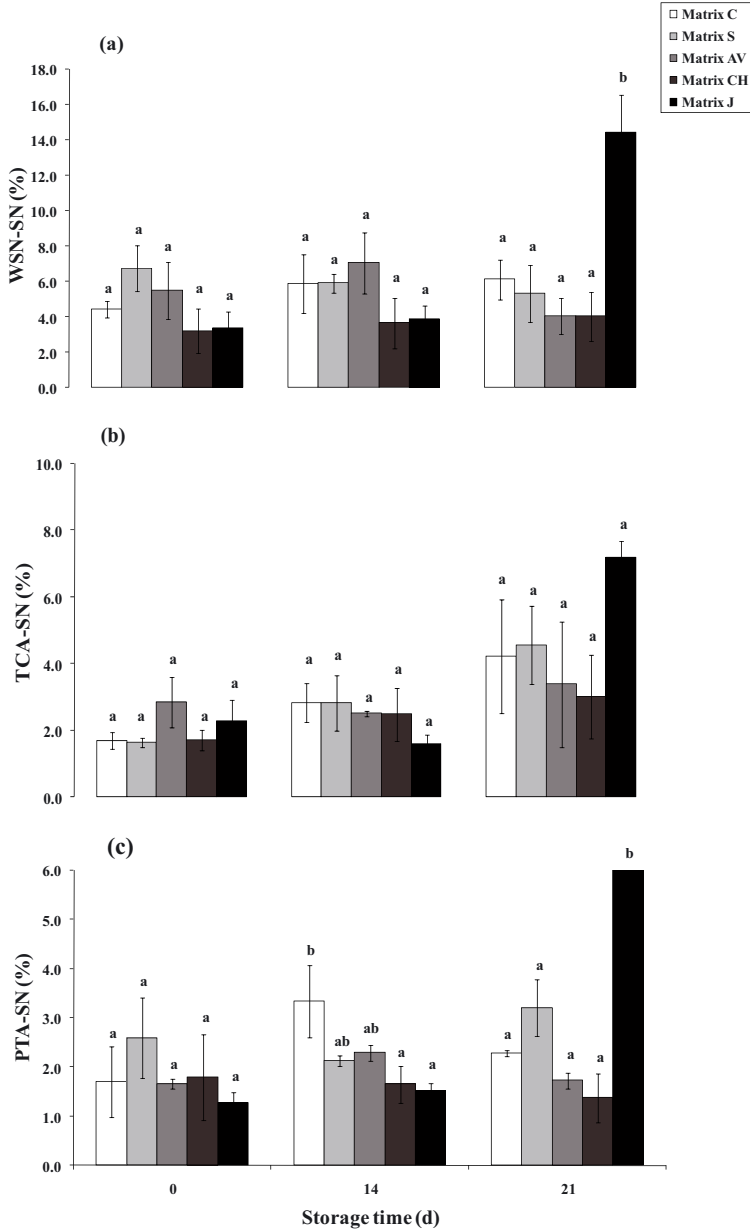
Figure 3. Evolution (average ± standard deviation), throughout storage at 7 °C, of proteolysis in whey cheese matrices measured as: (a) water-soluble nitrogen (WSN), (b) TCA-soluble nitrogen (TCA-SN) and (c) PTA-soluble nitrogen (PTA-SN). Means for matrices, at the same storage time, marked with the same letter, do not differ from each other ($P > 0.05$).
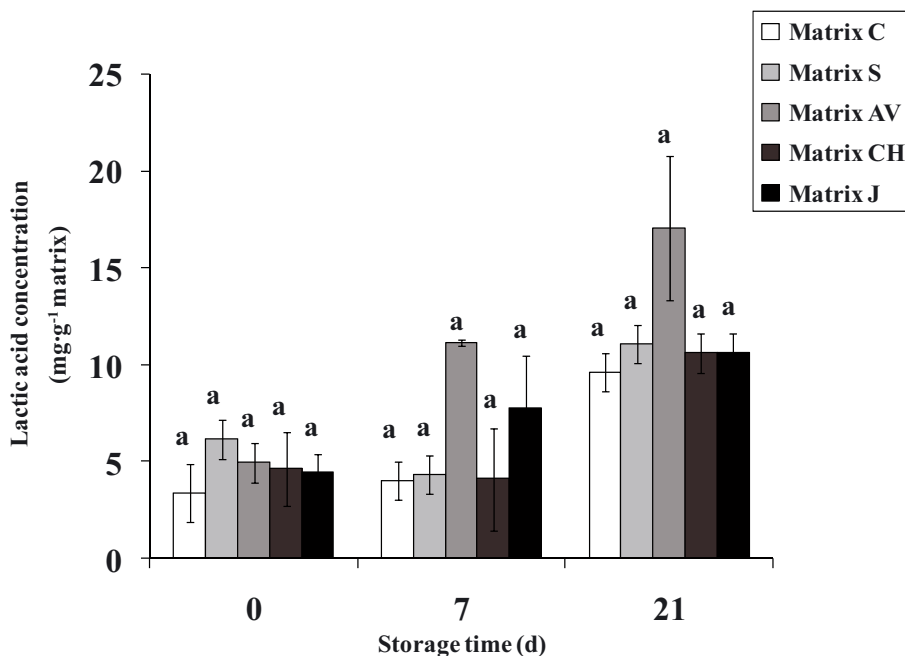
Figure 4. Evolution (average ± standard deviation), throughout storage at 7 °C, of glycolysis in whey cheese matrices measured as contents of lactic acid. Means for matrices, at the same storage time, marked with the same letter, do not differ from each other ($P > 0.05$).

A similar proteolysis level was also obtained for Cheddar cheeses manufactured with milk inoculated with a similar *L. paracasei* strain, and stored at low refrigeration temperatures (4 °C) for a period of 8 wk [28]. These strains are generally assumed to possess a relatively low level of caseinolytic activity, coupled with a high level of peptidolytic activity [9, 39].

Whey proteins are in a denatured and aggregated form in whey cheese. This state hampers full access of, and action by bacterial peptidases. In addition, there was no decrease in strain viability, so one concluded that no cell lysis had occurred. Hence, no extra release of intracellular peptidases into the matrix took place [17]. Finally, the matrices were manufactured without addition of rennet or coagulant, so no initial breakdown of substrate proteins

took place at all. Therefore, no reason exists to believe that denatured whey proteins are more susceptible to proteolytic attack than their soluble counterparts.

Peptides and free amino acids released throughout storage, notwithstanding their low levels, may be important as growth promoters of *L. paracasei*. Release of free amino acids may also contribute, to a small extent, to development of flavours that would possibly be perceived in a more refined sensory analysis.

3.5. Glycolysis

Storage time, but not type of matrix, proved an important technological parameter ($P < 0.05$) for content of lactic acid (Fig. 4). By 0 d, lactose concentrations

were 21–30 mg·g^{-1} (Tab. II); lactose concentrations were not affected by the type of additive used ($P > 0.05$).

Lactic acid was found at time zero. This was expected because the bacterial strain inoculum was made in skim milk, which contains lactose that was accordingly metabolised into lactic acid, and added to the whey matrices when the inoculum was homogenised. Note that control matrices also contain lactic acid because (as explained above) the addition of inoculum was mimicked by skim milk deliberately acidified with lactic acid. The concentration of this acid increased throughout storage time; the lowest value observed was in matrix S by 21 d (Fig. 4). The presence of additives increased the production of lactic acid by the probiotic strain, especially in matrix AV.

Lactose is obviously the primary substrate for acid production, as a lactic acid bacterium was the sole microorganism at stake. *Lactobacillus paracasei* is known to be a facultative heterofermentative lactobacillus, as it converts lactose into lactic acid, acetic acid and carbon dioxide. In all experimental matrices, initial lactose was partially converted into lactic acid, whereas generation of acetic acid was almost negligible (results not shown). Again, several parameters may have influenced the metabolism of this strain; for example, storage at 7 °C, time of storage and absence of oxygen, which may affect release of acetic acid, but not of lactic acid. Furthermore, sucrose was present in all matrices but matrix C, and can be degraded into fructose and glucose (data not shown), whereas glucose may in turn be converted into lactic acid.

Production of lactic acid accounts for the acidification conveyed by Figure 2 (and discussed before). However, production of lactic acid also fulfils an antimicrobial role, as it inhibits endogenous growth of postprocessing contaminating bacteria (e.g. pathogens).

3.6. Textural profiles

Our experimental manufacture of whey matrices encompassed slight modifications of a protocol reported previously [24]. In order to improve softness and increase yield, milk was added only upon whey protein precipitation (at 87–90 °C) – thus paralleling the more traditional recipe followed in manufacture of Serra da Estrela Requeijão. Both the inoculum and the additives were previously homogenised within the matrices, using optimal speed and equipment configuration (results not shown).

Different patterns were observed among matrices (Fig. 5). Softness was low and it decreased (in our case) in matrices C and CH ($P > 0.05$) as storage time elapsed, whereas this parameter increased in matrices S, AV and J ($P > 0.05$), as apparent from inspection of Figure 5a. In the case of matrix J, softness was higher towards the end of storage, unlike what happened in matrix C. Incorporation of additives led to increase in softness, and statistically significant differences were found only between matrices AV and CH ($P < 0.05$). Increase in softness may be related to the increase in acidity originated by the higher proteolytic activity detected in these matrices.

Matrices C and CH were the only ones that changed firmness with time ($P < 0.05$). By 7 d of storage, matrix C was harder than its counterparts, and by the end of storage this was observed for matrix CH (Fig. 5b). The degree of proteolysis relates to textural parameters, especially hardness; and acidification promotes a pH decrease, which in turn promotes release of calcium ions; these changes in free calcium concentration levels eventually account for fragile and fragmented matrices [38]. In matrix CH, lower proteolysis degrees were detected and it was rated as firmer than the others. Matrix J suffered the highest proteolysis and acidification extents, and
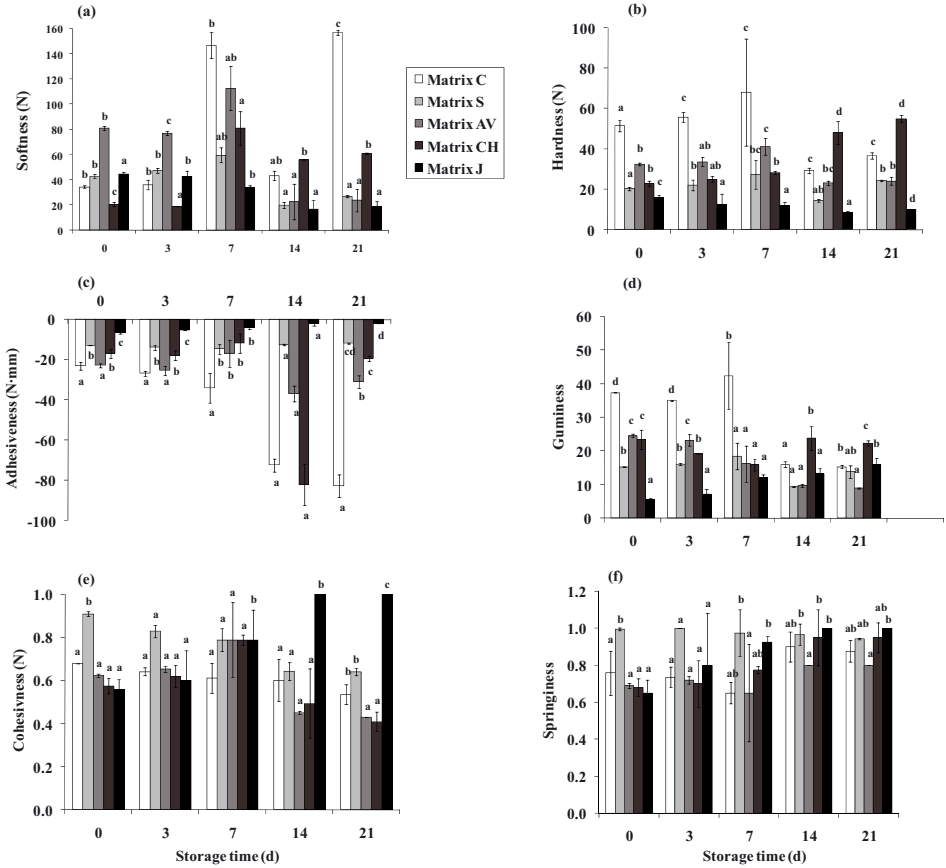
Figure 5. Evolution (average ± standard deviation), throughout storage at 7 °C, of texture in whey cheese matrices measured as: (a) softness, (b) hardness, (c) adhesiveness, (d) gumminess, (e) cohesiveness, and (f) springiness. Means for matrices, at the same storage time, marked with the same letter, do not differ from each other ($P > 0.05$).

in turn was less firm than the remaining matrices.

Adhesiveness was statistically different between matrices ($P < 0.05$), and increased in the case of matrices C, AV and CH until 14 d of storage (Fig. 5c). The highest value was found for matrix C, and the lowest for matrix J.

Gumminess decreased only for matrix C, and increased for matrix J. For the other matrices, this parameter remained essentially constant throughout storage time.

The highest value was found for matrix CH by the end of storage, whereas the lowest was found for matrix AV. Changes in gumminess were statistically significant among all matrices ($P < 0.05$) (Fig. 5d).

Cohesiveness decreased in all matrices ($P < 0.05$), except for J (Fig. 5e).

In all matrices but S, elasticity underwent similar changes, which were statistically significant ($P < 0.05$), versus storage time (Fig. 5f). This parameter

Table III. Sensory scores of acceptance (average ± standard deviation) of whey cheese matrices, throughout storage at 7 °C: (C) control, (S) with added sugar, (AV) with added sugar and aloe vera, (CH) with added sugar and chocolate, and (J) with added strawberry jam.

Storage time (d)	Matrix				
	C	S	AV	CH	J
3	7.16 ± 1.04	8.01 ± 0.86	7.28 ± 0.75	6.06 ± 1.87	6.13 ± 1.89
14	6.63 ± 1.37	5.48 ± 0.97	4.83 ± 1.02	4.05 ± 1.39	3.73 ± 2.27

was influenced by the type of additive ($P < 0.05$).

Experimental matrices were manufactured so as to parallel the original Requeijão. This Portuguese speciality whey cheese is considered a soft cheese in its original form, as moisture content lies in the range 48–80% (w/w). However, preliminary homogenisation of the inoculum and additives produced even softer matrices. Softness and hardness (firmness) are directly correlated with each other: harder matrices are also less soft. On the other hand, additives play important roles in the two aforementioned textural parameters. By the time of manufacture, matrix CH was the softest, but as time elapsed this matrix became less soft (Figs. 5a and 5b). Chocolate was incorporated in the form of powder and fibre; its components (including fibre) may have absorbed water throughout storage time, thus turning them harder (and thus less soft). Conversely, the matrices in which the additives were incorporated in liquid form, i.e. aloe vera and jam, were softer and thus less hard.

3.7. Sensory profiles

The results of organoleptic assessment of the various experimental whey cheese matrices are depicted in Table III. Matrices containing sugar, and sugar and aloe vera, received the best scores, by 3 d of storage.

Sugar addition appeared to increase the sensory scores attributed (Tab. III). The panel emphasised that matrices with added

aloe vera were perceived as particularly fresh. As storage time elapsed, all matrices tended to slightly decrease the scores they received, especially those with added jam and chocolate. By 14 d of storage, all matrices decreased their levels, and the control matrix (C) was the best scored, since lactic acid was present at lower levels. The presence of additives increased the metabolic activity, and led to higher production of lactic acid, especially in matrix J. This shortcoming may be effectively addressed via use of an alternative low-acid producer, yet probiotic strain (for example, belonging to the *Bifidobacterium* genus), or additives with explicit anti-acid capacity (for example, sodium citrate or bicarbonate). As a basis for selection, strains which cannot grow at 15 °C (a constraint that excludes *L. casei*, *L. rhamnosus* and *L. plantarum* groups), and which have low lactase and/or invertase activities, would be good candidates. In a previous work [24], matrices inoculated with *Bifidobacterium* strains exhibited a slower acidifying profile. Another solution would be to use jam kept apart from the whey cheese matrix, as this additive also contributes to acidification.

Texture was always pointed out as a positive feature of these matrices. The panel suggested specifically that all matrices were rather creamy; therefore, the (unwanted) presence of granules was eventually overcome, via homogenisation of the inocula and additives upon incorporation thereof.

It might be argued that use of a control without a probiotic would have permitted

one to assess whether addition of probiotics has itself a detrimental effect upon the sensory profile (including texture). Since the main objective of this research work was to assess the influence of additives on probiotic matrices, no experimental matrices without a probiotic strain were manufactured. Nevertheless, it is possible to effectively comment on the influence of such a probiotic strain in the matrices produced without additives. These matrices (C) exhibit no significant changes in proteolysis, and extent of release of lactic acid doubles only by 21 d of storage, which means that incorporation of additives influences their metabolic activity. In terms of texture, these matrices were less soft than the others. They were also more adhesive, but no changes in cohesiveness or springiness were detected during storage time. Such changes in texture were associated specifically with the strain and not with the additive, and contributed positively to the sensory scores, which remained essentially constant up to 14 d. Finally, the matrices were better scored than salted ones (Madureira et al., unpublished). Sweet additives thus proved to be a good alternative, in attempts to increase the perceived texture of these matrices. This appears to be an advantage, should they be intended for consumption as a dessert.

4. CONCLUSIONS

The viable cell numbers of *L. paracasei*, as well as acidification and pH profiles, were not influenced by the type of additive incorporated. No contamination was detected in any matrix during the course of the experiments. All matrices underwent acidification during storage, which was accounted for by lactic acid produced by the inoculated strain, whereas the highest values were found in matrices containing additives. As expected, the proteolytic activity measured was low, owing to the relatively low temperatures used for storage, the short storage time, and especially the nature of the matrix. Nevertheless, the highest proteolytic activity was found in matrices with jam, which influenced texture: matrix CH, which underwent lower proteolysis, was firmer, whereas matrix J was in turn softer, less adhesive and more cohesive. Incorporation of additives appears to increase softness, and to decrease hardness of matrices. Sensory assessment of matrices with additives was rather good compared with that of the control. However, the longer the storage time, the more preferred control matrices. The presence of additives is determinant for evolution of proteolysis and glycolysis, and both these activities are important for textural and sensory acceptance of matrices.

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