Influence of Coryneform Cultures Isolated from the Surface of a Farmhouse Smear-Ripened Cheese on the Ripening of Cheddar Cheese

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ABSTRACT

Smear-ripened cheeses are characterized by a layer of yeasts and bacteria on cheese surfaces. The microbial composition of smear of these cheeses is dominated by salt-tolerant yeasts and Gram-positive bacteria, particularly coryneforms and Staphylococci. Cheddar cheeses were manufactured using four strains of faculatively anaerobic coryneform bacteria (*Corynebacterium casei*, *C. flavescens*, *C. mooreparkense* and *Microbacterium gubbeenense*), these bacteria isolated from the surface of an Irish farmhouse smear-ripened cheese. Five experimental cheeses (containing isolated cultures in addition to the control cheese) were manufactured in two trials at inoculum levels of 10^7 cfu/g. In the experimental cheeses, counts of isolated cultures decreased from 10^7 to 10^6 cfu/g in two trials, while numbers of starter bacteria decreased from 10^9 cfu/g to 10^6-10^7 cfu/g at the end of ripening, on the other hand non-starter lactic acid bacteria started growing after 4 months of ripening and reached10^5 -10^6 cfu/g. Proteolysis during ripening, measured by urea-polyacrylamide gel electrophoresis and levels of water-soluble nitrogen. The results showed no differences between the control and experimental cheeses in both trials. However, the experimental cheeses had higher levels of total free amino acids than did their corresponding controls throughout ripening, as well as differences in profiles of individual free amino acids. Reverse-phase (RP)-HPLC of the 70 ml 100ml^{-1} ethanol-soluble and -insoluble fractions showed differences between control cheese and experimental cheeses. Principal component analysis of chromatographic data from ethanol soluble and insoluble fractions of the cheeses after 4 and 6 months of ripening clearly differentiated between the control and experimental cheeses in first and second batches.

Key words: corynebacterium, microbacterium, NSLAB, proteolysis, FAA, RP-HPLC.

INTRODUCTION

There has been considerable interest in using defined strains of non-starter lactic acid bacteria "NSLAB" as isolated cultures to accelerate and improve flavour and texture during cheese ripening (El-Soda, 1993, McSweeney *et al*., 1994, Tobin, 1999). All adjunct-contaning cheeses developed higher levels of free amino acids than the control cheese.

In addition to the NSLAB, many other bacteria have been used as isolated cultures. Law (1987) accelerated the development of Cheddar cheese flavour by adding cell-free extract of Brevibacteria, which produce large amounts of methanethiol. A combination of citrate-positive lactobacilli and *Brevibacterium linens* improved both flavour and texture of cheese (Jensen, 1998).

The surface of smear-ripened cheeses is covered by a layer of yeasts and bacteria which have a strong effect on the appearance and the flavour of these cheeses (Bockelmann, 2002). The composition of the surface flora of commercial smear cheeses still depends on the specific in-house microflora that becomes established on cheese surface during manufacture. Adventitious species of *Brevibacterium*, *Arthrobacter*, *Staphylococcus*, *Micrococcus* and *Corynebacterium* are the dominant microorganisms in bacterial surface-ripened cheese at the end of ripening (Brennan *et al*., 2004).

The objectives of the present study were to determine the effects of *Corynebacterium casei*, *C. flavescens*, *C. mooreparkense* and *Microbacterium gubbeenense* cultures, which isolated from farmhouse smear-ripened on proteolysis in Cheddar cheese during ripening.

MATERIALS AND METHODS

Cultures

Lactococcus lactis subsp. *lactis* 303 was obtained from the Microbiology Department, University College, Cork. Frozen cultures (1% v/v) were propagated at 30°C for 16 hr in autoclaved (110°C, 10 min) reconstituted skim milk powder (100 g L-1). Two transfers were made prior to cheesemaking, bulk cultures were grown for 16 hr at 21°C and were used as starters at levels of 2% (v/v).

Four strains of facultatively anaerobic coryneform bacteria (*Corynebacterium casei* DPC 5298, *C. flavescens*, *C. mooreparkense* DPC 5310 and *Microbacterium gubbeenense*) isolated from the surface of an Irish farmhouse smear-ripened cheese were tested as isolated cultures. All the strains were obtained from the culture bank of the National Dairy Products Research Centre, Moorepark, Fermoy, Co. Cork. Each strain was grown in 50 ml MRS broth (Oxoid Ltd., UK) at 30°C overnight. Sufficient culture was then added to cheesemilk to give an inoculum of 10^7 cfu/ml milk.

Cheese manufacture

Cheddar cheese was made on two occasions for both first and second trials in 20 L open vats from pasterurized (74°C × 15 s) milk. Five cheeses were made on each occasion using a standard protocol (Kosikowski, 1982).

Compositional analysis

Cheese samples were analysed after one month of ripening for moisture (oven drying at 102°C, IDF, 1982), total protein (macro-Kjeldahl, IDF, 1964), fat (Gerber method, Bradley *et al*., 1992) and salt (Fox, 1963).

Bacteriological analysis

Microbiological counts were performed on the cheese-milk before and after inoculation, and on the cheeses during ripening at 1 day and 1, 2, 4 and 6 months. Lactococci were enumerated on duplicate plates of M17 agar (Merck, Germany) which were incubated aerobically at 30°C for 48 hr (Terzaghi & Sandine, 1975). Non-starter lactic acid bacteria (NSLAB) were enumerated on duplicate plates of Rogosa agar (Merck, Germany) which were incubated at 30°C for 5 days under anaerobic conditions (Gas-pack System, BBL, Oxoid, UK). Isolated cultures were enumerated on selective media (Cheese Ripening Bacteria Medium, CRBM) (Denis *et al*., 2001).

Assessment of proteolysis

Samples (200 g) were taken periodically during ripening for the assessment of proteolysis and frozen at 20°C until analysed. The pH of 4.6-insoluble and soluble fractions of the cheese samples were prepared by a modification of the procedure of Kuchroo & Fox (1982) as described by Sousa & McSweeney (2001). The pH of 4.6-soluble fraction was sub-fractionated into 70 ml 100 ml^{-1} ethanol-soluble (small, hydrophilic peptides) and insoluble (large, hydrophobic peptides) fractions as described by Lynch *et al*. (1996).

Urea-polyacrylamide gel electrophoresis (PAGE, 12.5% T, 4% C, pH 8.9) of the pH 4.6-insoluble fractions of the cheeses were performed using a Protean II XI vertical slab-gel unit (Bio-Rad Laboratories Ltd., UK) according to the method of Andrews (1983) as modified by Shalabi & Fox (1987). The gels were stained directly with Coomassie Brillant Blue G250, as described by Blakesley & Boezi (1977).

Peptide profiles of the ethanolinsoluble and soluble fractions of the pH 4.6-soluble fractions were determined by RP-HPLC using a Varian System (Varian Associates Inc., USA). Samples were prepared before analysis as described by Sousa & McSweeney (2001).

Total levels of free amino acids in the pH 4.6-soluble fractions of cheese were determined by the trinitrobenzenesulphonic acid (TNBS) method of Polychroniadou (1988) and levels were calculated with respect to a standard curve prepared using leucine. Individual free amino acids were determined after 6 months of ripening using the method described by Fenelon *et al*. (2000).

Statistical analysis

Data from RP-HPLC chromatograms of the ethanol-soluble and -insoluble fractions of the cheeses were pre-processed using a logistic function (Pirano *et al*., 2004) and the resulting categories were analysed using multivariate statistical techniques to evaluate the effect of each of the five cultures on ripening. Principal component analysis (PCA) was performed using a covariance matrix. Statistical analysis was performed using SPSS, Version 10.0 for Windows 98 (SPSS Inc., USA).

RESULTS AND DISCUSSION

Cheddar cheese made with cultures added at levels of 10^7 cfu /ml milk

Composition

The composition of Cheddar cheeses at 1 month of ripening are shown in Table (1). The composition of control and experimental cheeses were similar. Compared to commercial Cheddar cheese, all cheeses had high moisture and slightly lower salt content but were similar to Cheddar cheese made previously on a pilot-scale (e.g., Shakeel-Ur Rehman *et al*., 2000).

Microbiological analysis of cheese

The numbers of *Corynebacterium* and Microbacterium strains in the control and experimental cheeses during ripening are shown in Fig. (1). The counts at day 1 of ripening were~ 5-7 × 10^7 cfu/g. However, this population declined slightly after 1 month of ripening for cheese made with C. casei, *C. mooreparkense* and M. gubbeenense to 10^6 cfu/g while numbers of *C. flavescens* remained constant during ripening.

Enumeration of starter in experimental cheeses (Fig. 2), showed all cheeses contained approximately 10^9 cfu/g lactococci cfu/g at 1 day of ripening, and that the number of viable starter bacteria declined slightly after 4 months of ripening to 10^7-108 cfu/g, starter counts reached 10^6-10^7 cfu/g after 6 months of ripening, although late in ripening, enumerating starter bacteria on LM17 agar becomes less accurate due to the growth of NSLAB on this medium which is used to enumerate starter organisms.

Table 1: Compositional parameters (%) at 1 month of ripening of Cheddar cheese made with added cultures of *Corynebacterium* sp. or *Microbacterium* sp.*

Isolated Cultures	Moisture	Protein	NaCl	Fat
C. casei	40.47 ±0.5	23.50 ±0.18	1.13 ±0.12	31 ±0.29
C. flavescens	40.46 ±0.14	23.70 ±0.06	1.19 ±0.17	32 ±0.23
C. mooreparkense	40.22 ±0.28	23.70 ±0.24	1.20 ±0.15	32 ±0.64
M. gubbeenense	40.16 ±0.02	23.60 ±0.28	1.19 ±0.15	32 ±0.60
Control	40.48 ±0.07	23.60 ±0.25	1.18 ±0.21	31 ±0.46

*Results presented as mean ± standard deviation of triplicate analyses.

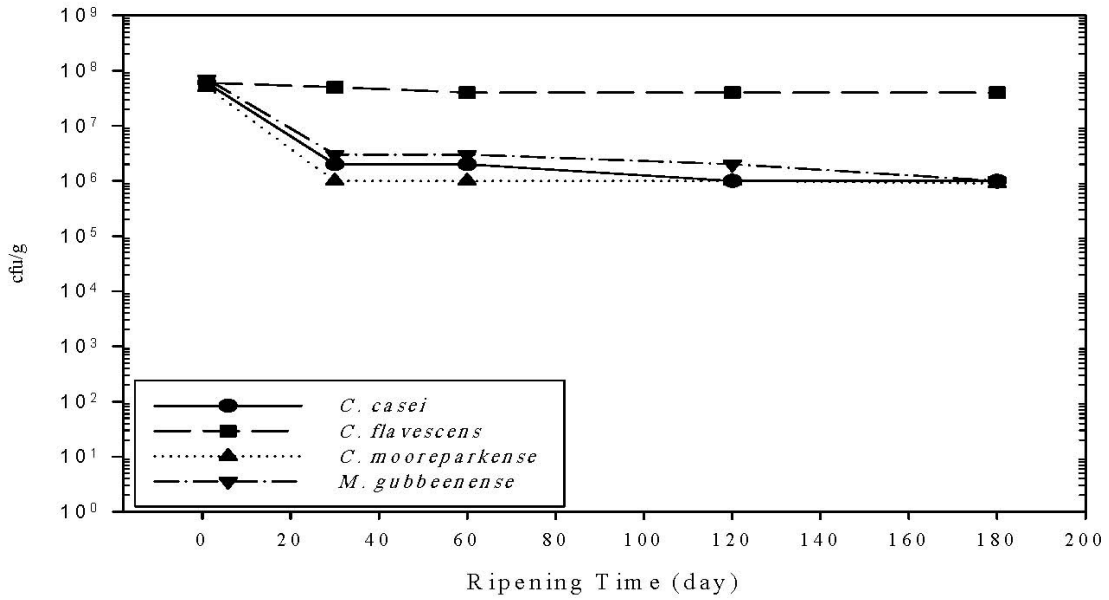

Fig. 1: Counts of isolated coryneforms during ripening, as enumerated on Cheese Ripening Bacteria Medium (CRBM) agar, for Cheddar cheese made without (control) or with added cultures of *Corynebacterium* sp. or *Microbacterium* sp.

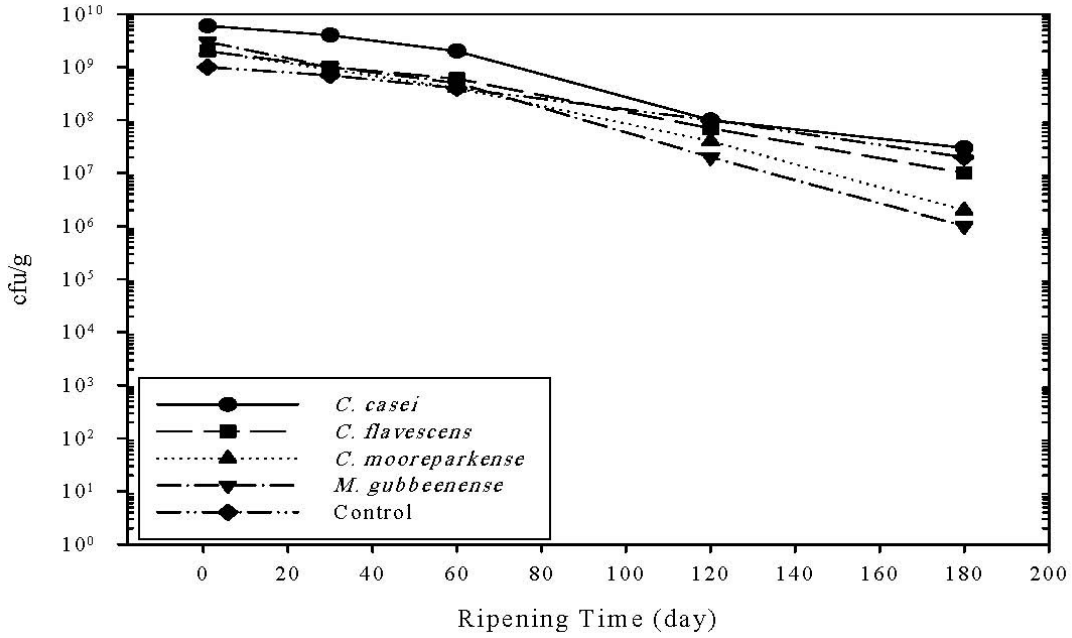

Fig. 2: Counts of lactococcal starter bacteria during ripening, as enumerated on LM 17 agar, for Cheddar cheese made with added cultures of *Corynebacterium* sp. or *Microbacterium* sp.

NSLAB remained <1 cfu/g for 2 months of ripening (Fig. 3). After 4 months of ripening, the numbers of NSLAB were between 10^4-10^5 cfu/g and reached to 10^5-10^6 cfu/g in all cheeses after 6 months of ripening.

Assessment of proteolysis

Higher increases in FAA content were observed in the experimental cheeses than in control cheese as ripening progressed (Table 2). A greater increase in the total FAA was observed in cheeses made with M. gubbeenense followed by *C. mooreparkense* and C. casei as isolates, while the control and the cheese made with *C. flavescens* had similar and the lowest levels of FAA.

The profiles for individual amino acids of Cheddar cheeses made with and without isolates after 6 months of ripening are shown in Fig. (4).

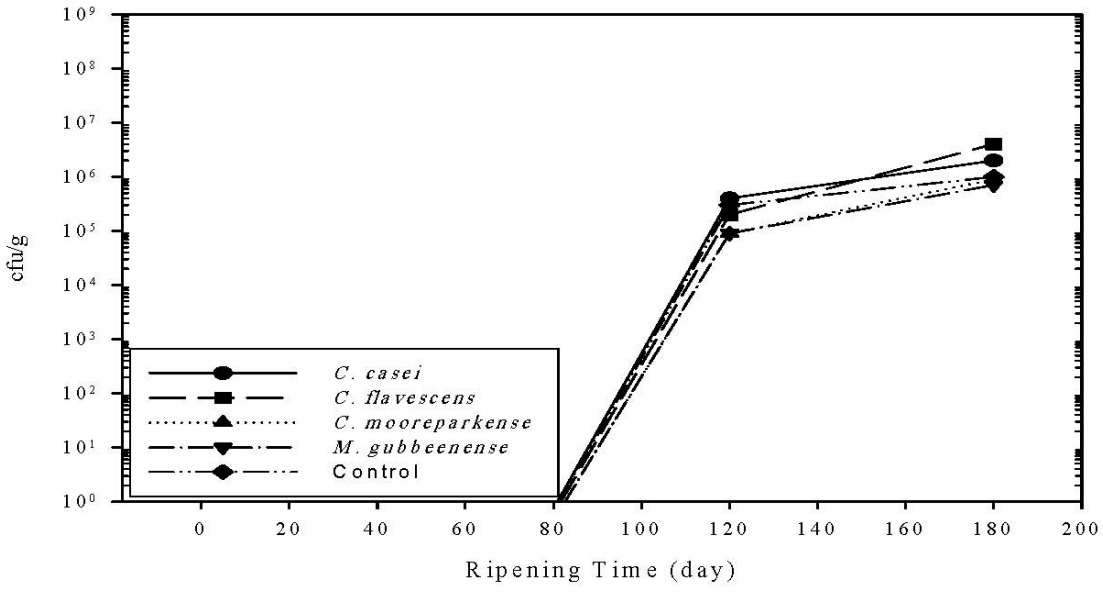

Fig. 3: Counts of non-starter lactic acid bacteria (NSLAB) during ripening, as enumerated on Rogosa agar, for Cheddar cheese made with added cultures of *Corynebacterium* sp. or *Microbacterium* sp.

Table 2: Total levels of free amino acids (±standard deviation, expressed as mg Leu. per g cheese) throughout ripening of Cheddar cheese made with added cultures of *Corynebacterium* sp. or *Microbacterium* sp.*

Ripening Time (day)	Control	*C. casei*	*C. flavescens*	*C. mooreparkene*	*M. gubbeenense*
1	3.6 ±0.10	3.4 ±0.15	3.4 ±0.29	3.6 ±0.15	3.4 ±0.06
30	7.3 ±0.12	7.7 ±0.27	7.3 ±0.21	7.5 ±0.06	7.6 ±0.53
60	11.8 ±0.32	12.2 ±0.21	11.9 ±0.17	11.8 ±0.29	12.1 ±0.17
120	14.9 ±0.27	16.0 ±0.21	15.1 ±0.21	15.0 ±0.27	15.8 ±0.06
180	16.6 ±0.15	18.4 ±0.15	16.8 ±0.60	19.8 ±0.31	22.5 ±0.43

*Results presented as mean ± standard deviation of triplicate analyses.

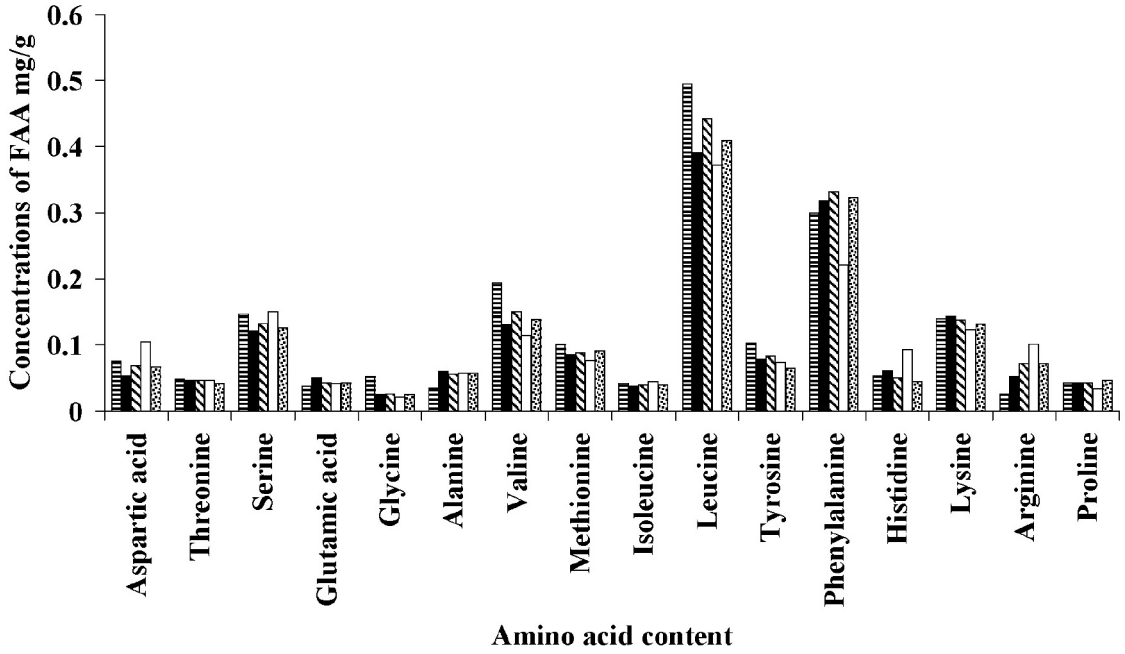

Fig. 4: Concentration of individual free amino acids in 6 months-old Cheddar cheese made (from left to right) without or with strains of *C. casei* ▤, *C. flavescens* ■, *C. mooreparkense* ▧, *M. gubbeenense* □ and the control ▩.

After 6 months, concentrations of Ser, Val, Leu, Phe, and Lys were presented at high levels in all cheeses. Concentrations of Ser and Tyr were higher in the experimental cheeses than in the control, on the other hand the concentrations of Val, Asp and Leu were higher in cheeses containing C. casei and *C. mooreparkense* than in the control.

Score plots obtained from PCA of the RP-HPLC data of the ethanol-soluble fractions after 4 and 6 months of ripening are shown in Fig. (5a, b). The PCA separated the cheeses after 4 months of ripening on the basis of isolates added, cheeses containing C. casei, *C. flavescens* in addition to the control cheese were located on the positive side of PC2 and cheeses containing *C. mooreparkense* and M. gubbeenense were located separately on the negative and positive sides of PC1, respectively. On the other hand, after 6 months of ripening the control cheese was located separately on the positive side of PC1 while cheeses containing C. casei and *C. flavescens* grouped together on the negative side of PC1 and other cheeses were located separately.

The scores obtained from PCA of peak height data from the RP-HPLC chromatograms of the ethanol-insoluble sub-fractions of cheeses after 4 and 6 months of ripening are shown in Fig (6 a, b). After 4 months of ripening, all cheeses were well separated by PCA, while after 6 months of ripening, all cheeses were also well separated, but cheeses containing C. casei and *C. flavescens* were grouped close to each other, and the control was located separately with high and low value of PC2, respectively.

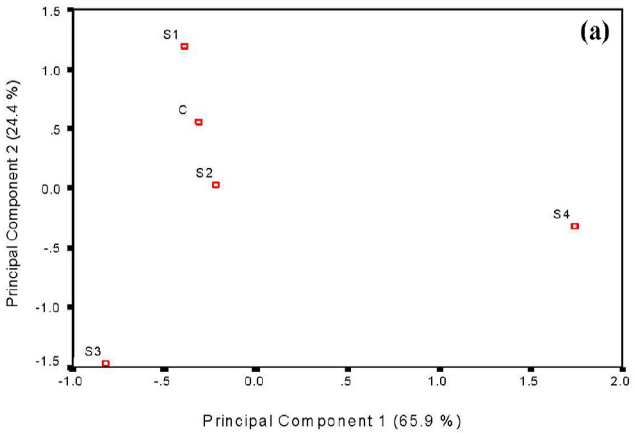

Fig. 5. Score plot obtained by principal component analysis (PCA) of the peak heights of reverse-phase HPLC chromatograms of the ethanol-soluble fractions from 4 months (a) and 6 months (b)-old Cheddar cheeses made without (control) C or with isolated cultures of coryneforms: (S1) *C. casei*, (S2) *C. flavescens*, (S3) *C. mooreparkense*, (S4) *M. gubbeenense*

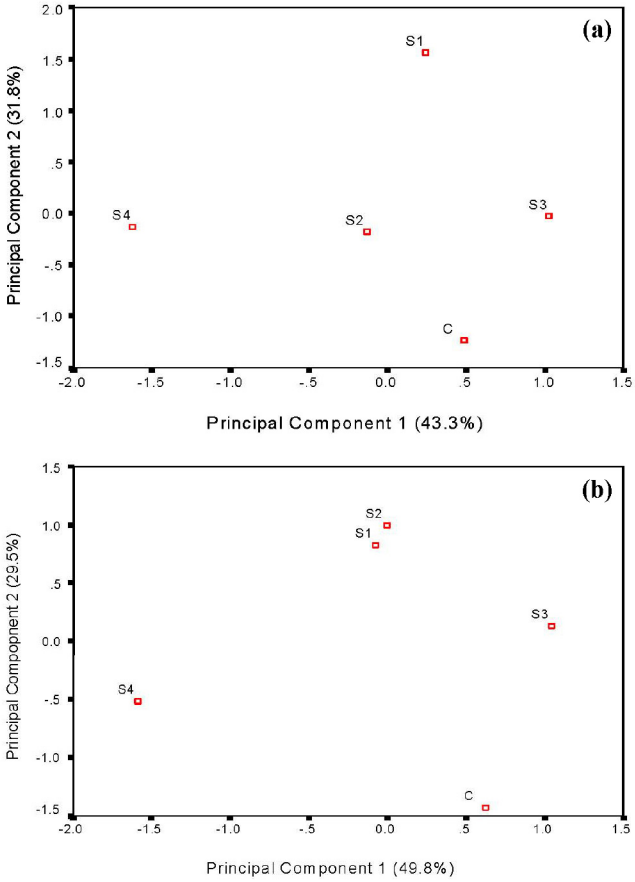

Fig. 6. Score plot obtained by principal component analysis (PCA) of the peak heights of reverse-phase HPLC chromatograms of the ethanol-insoluble fractions from 4 months (a) and 6 months (b)-old Cheddar cheeses made without (control) C or with isolated cultures of coryneforms: (S1) *C. casei*, (S2) *C. flavescens*, (S3) *C. mooreparkense*, (S4) *M. gubbeenense*

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تأثير سلالات من بكتيريا Coryneform معزولة من سطح جبن منضج سطحياً والمصنع في مزارع الريف الأيرلندي على إنضاج جبن التشدر

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تم صناعة جبن التشدر باستخدام أربع سلالات من بكتيريا الـ Coryneform (*Coryneform casei*, *C. mooreparkense*، *C. flavescens*, *Microbacterium gubbeenense*) المعزولة من سطح جبن منضج سطحيا والمصنع بمزارع الريف الايرلندي " وهو جبن يتبع الأجبان المصنعة بالأنزيم وتتميز بوجود طبقة من الخمائر والبكتيريا على سطحها" حيث أستخدم تركيز ١٠٧ خلية بكتيرية / مل من لقاح الـ Coryneform وهي بكتيريا لا هوائية اختيارية وذلك بهدف دراسة تأثيرها على تحليل البروتين أثناء إنضاج جبن التشدر. تم استخدام هذه السلالات الأربع في صناعة جبن التشدر " كل سلالة خاصة بصناعة جبن " بالإضافة إلى جبن المقارنة" أو الكونترول في كل معاملة، وقد تم صناعة هذه الأجبان مرتين تحت نفس ظروف التصنيع.

بينت نتائج الدراسة أن تركيز اللقاح انخفض اثناء عملية ألانضاج من ١٠٧ إلى ١٠٦ خلية بكتيرية /جم، وعلى الجانب الآخر بدأت بكتيريا حمض اللاكتيك " غير البادئ " في النمو بعد ٤ شهور من الإنضاج وتراوحت بين ١٠٥ - -١٠٦ خلية بكتيرية / جم في نهاية عملية ألانضاج (٦ شهور).

تم قياس التحلل البروتيني أثناء الإنضاج باستخدام جهاز الهجرة الكهربائية Urea-PAGE، حيث لوحظ انه لا توجد اختلافات جوهرية بين جبن المقارنة "الكونترول" والأجبان الملقحة أثناء ألانضاج، أعطت الاجبان الملقحة بالسلالات مستويات عالية من الأحماض الأمينية الكلية مقارنة بجبن المقارنة بالاضافة إلى وجود اختلافات بين كل من جبن المقارنة والاجبان الملقحة عند تقدير الأحماض الأمينية الحرة وقد تم تقدير الجزء البروتيني الذائب وغيرالذائب في الكحول باستخدام جهاز الفصل الكروماتوغرفي عالي الكفاءة (RP-HPLC) والذي اظهر فروقاً اساسية بين جبن المقارنة والاجبان الملقحة، وبتحليل هذه البيانات باستخدام نظام Principle Component Analysis (PCA) بعد ٤ و٦ شهور من الانضاج ظهرت اختلافات واضحة بين جبن المقارنة والاجبان الملقحة.