J. Food and Dairy Sci., Mansoura Univ., Vol. 5 (8): 549 - 560, 2014

PROTEOLYSIS AND BIOGENIC AMINES FORMATION IN STERILIZED EDAM CHEESE CURD SLURRY INOCULATED WITH SOME PROBIOTIC STRAINS FORMULATIONS

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ABSTRACT

In this investigation, Sterilized Edam cheese curd slurry was inoculated with 1% *Lactococcus lactis subsp. lactis* KF147 and served as control. The effect of added some probiotic strains formulations upon proteolysis pattern and biogenic amines (BAs) during incubation at 30 °C for 21 days. was also evaluated. Sterilized Edam cheese curds slurry were inoculated with 1% of the following formulations: *Propionibacterium shermenii* PS-4 + *Bifidobacterium bifidum* DSM 20082 (1:1), *Propionibacterium shermenii* PS-4 + *Lactobacillus acidophilus* ATCC4356(1:1), or *Propionibacterium shermenii* PS-4 + *Bifidobacterium bifidum* DSM 20082 + *Lactobacillus acidophilus* ATCC4356 (1:1:1). Results showed no significant influence of any formulation of probiotic bacteria on total solids, salt and fat contents of sterilized Edam cheese curd slurry. However, inoculation of sterilized Edam curds slurry with probiotic strains formulations affected the pH and soluble nitrogenous compounds (S.N.C). The formulation consisting of *Propionibacterium shermenii* + *Bifidobacterium bifidum* + *Lactobacillus acidophilus* (1:1:1) showed the highest concentration (7.9 %) of water-soluble nitrogenous compounds at the end of incubation period. While inoculation included *Bifidobacterium bifidum*, a significant reduction in total BAs (447 - 37 mg/kg DW) was observed by 21 d Also, both histamine and tyramine was decreased from 84 - 25, and 359 - 6 mg/kg DW respectively.

Keywords: Curd slurry, probiotics, proteolysis, biogenic amines.

INTRODUCTION

Cheese ripening is a complex process that includes several biochemical reactions leading to cheese flavour and texture development, namely via glycolysis, proteolysis and lipolysis; proteolysis in particular is strongly influenced by the activity of microbial enzymes during ripening (Fox *et al.,* 1993).

Starter cultures account for a major portion of the microflora in young cheese curd; during ripening, starter numbers decrease owing to cell death and subsequent autolysis (Crow *et al.,* 1995). The potential of intracellular peptidases to hydrolyze peptides following autolysis indicates that this may be an important factor in cheese flavour development. Comprehensive experimental evidence suggests that adjunct cultures or non-starter lactic bacteria also play a role in cheese ripening (Peterson *et al.,* 1990; Rodriguez, 1998).

It is of particular interest, the use of cheese as carrier of probiotic strains owing to its mild pH and higher fat content, both of which favour survival when compared to fermented milks (Stratton *et al.,* 1991); this has been consubstantiated for such probiotic species as *Bifidobacterium bifidum*,

Lactobacillus acidophilus and *Lactobacillus casei* in curd slurry (Vinderola *et al.,* 2000), as well as *Enterococcus faecium* in yoghurt (Gardiner *et al.,* 1977) – whereas (Vinderola *et al.,* 2002) emphasized the interactions between mesophilic starter and probiotic cultures in cheese.

Biogenic amines (BAs) in cheese have a high impact on public health, since they are potentially toxic to humans at levels >100 mg kg^{-1}. These are low-molecular nitrogenous compounds formed chiefly via microbial decarboxylation of precursor amino acids (Halász *et al.,* 1994). So, they can serve as indicator of food quality Rauscher-Gabernig *et al.,* (2009). Due to the oxidation system encompassing monoamine and diamine oxidases, small amounts of BAs can be metabolized in the organism without any major impact on health – but ingestion of higher levels brings about serious impacts, e.g. release of adrenaline and noradrenaline leading to gastric acid secretion, increased cardiac output, migraine, tachycardia and high blood pressure (Shalaby, 1996; Loret, 2005); furthermore, a fraction of the population has histamine (HIS) intolerance (Maintz *et al.,* 2007).

The ability to decarboxylate amino acids is present in many genera of lactic acid bacteria (LAB) (Halász et al.,1994; Arena *et al.,* 2001) – and tyramine (TYR), HIS, putrescine and cadaverine (CAD) can be found to considerable levels, and other BAs to lesser amounts (Spano *et al.,* 2010). A few strains of lactic acid bacteria, e.g. *Lactococcus lactis* subsp. *lactis*, have exhibited decarboxylase activity (Rabie *et al.,* 2011a); however, lactobacilli have also been associated to release of HIS, TYR and putrescine (PUT), and enterocci to release of TYR (Bover-Cid *et al.,* 1999). The optimum pH of decarboxylases lie usually in the acidic region; therefore, fermented dairy products represent a suitable environment for formation of BAs (Halász *et al.,* 1994; McSweeney *et al.,* 2004) – and *Lactococcus lactis* is presumably responsible for BAs in cheeses at large where it are used as starter culture.

Little data are available on the occurrence of biogenic amines in Edam-type curd slurries. Therefore, the objective of this study was to provide information on the concentration of these components release, by ascertaining the effects of adding probiotic strains to the regular inocula of lactic acid bacteria.

MATERIALS AND METHODS

Materials:

Cow's milk

Cow's milk, containing 3.5 % fat and 12 % TS, was obtained from Dairy Technology Unit of Food Science Department, Faculty of Agriculture, Zagazig University (Egypt).

Starter and probiotic microorganisms cultures

Lactococcus lactis subsp. *lactis* KF147 and *Propionibacterium shermenii* PS-4 were obtained from Chr. Hansen Laboratories (Copenhagen, Denmark). *Bifidobacterium bifidum* DSM 20082 and *Lactobacillus acidophilus* ATCC4356 were obtained from Cairo Microbiological Center, MIRCEN, Faculty of Agriculture, Ain Shams University (Egypt). Stock cultures were

kept frozen at -70ºC in 30 % (v/v) glycerol. Prior to use, each strain was cultivated in MRS broth for 48 h, and underwent two consecutive transfers. When used as adjuncts, the aforementioned strains were inoculated at 0.1% into sterile 2 % fat milk, and incubated for 24 h at the appropriate temperature.

Preparation of Edam cheese curd slurry

Standardized cow's milk 2% fat was pasteurized at 72°C for 15 sec, cooled to 32°C, and transferred to a sterile cheese vat under a laminar flow hood. Approximately 100 L of milk was used to manufacture Edam cheese curd, following the procedure described by Scot (1979).

Cheese curd slurry was prepared as described by Farkye et al. (1995); it was salted at a rate of 2 %, and transferred aseptically into a sterile, wide mouth bottle, and sterilized at 121°C for 15 min. All aseptic cheese slurries were aseptically inoculated with 1 % of Edam cheese starter. One part of cheese slurry without added probiotic stains served as control. The other portions of cheese slurry were aseptically inoculated with 1% of each combination of *Propionibacterium shermenii* PS-4 + *Bifidobacterium* DSM 20082, or *Propionibacterium shermenii* PS-4 + *Lactobacillus acidophilus* and or *Propionibacterium shermenii* PS-4 + *Lactobacillus acidophilus*ATCC4356 + *Bifidobacterium* DSM 20082 (1:1:1). Each slurry preparation was replicated three times, using freshly made Edam cheese curd slurry. Cheese slurry bottles were incubated at 30°C for 7, 14 and 21 days.

METHODS:

Assessment of proteolysis

The pH 4.6-water soluble nitrogen (WSN), 12% trichloroacetic acid soluble nitrogen (TCA-SN), Non protein nitrogen (NPN) and Phosphotungestic acid soluble nitrogen PTA-SN amino acid nitrogen (AN) were determined as described by (Gripon *et al.,* 1975).

Evaluation of flavour intensity

Flavour intensity of Edam cheese curd slurry with or without probiotic bacteria was evaluated as described by (King *et al.,* 1979).

Extraction of biogenic amines

Biogenic amines in curd slurry samples were extracted as originally described by (Simon-Sarkadi and Holzaphel., 1994), and later improved by (Rabie *et al.,* 2011b). To extract BAs, 10 mL of 10 % trichloroacetic acid (TCA) was added to 3 g-samples of cheese, and the mixture was shaken for 1 h using a Laboshake (Gerhardt Ls 500i, Germany). The extract was filtered through Whatman No.1 filter paper. To remove the fat content, the samples were kept at -20 °C for 1 day, and then centrifuged (MLW, T 24, Germany) at 7000 ×g for 15 min. Supernatants were collected and filtered through a 0.25-µm membrane filter (Nalgene, USA).

Quantification of biogenic amines

The analysis of BAs was performed with an AAA 400 amino acid analyser (Ingos, Czech Republic), equipped with an Ostion LG ANB ion-exchange column. Colorimetric detection was accomplished at 570 and 440 nm, after post-column derivatization with ninhydrin (Csomos *et al.,* 2002). The operating parameters used were summarized at Table 1.

Table 1: Analytical conditions utilized for quantitation of biogenic amines.

Items	Feature	Value
Column	length	7 cm
	diameter	0.37 cm
	temperature	60°C
Eluent	flow rate	0.30 mL min^{-1}
	run time	101 min
Reagent	flow rate	0.20 mL min^{-1}
	temperature	121 °C
Injection	sample volume	100 µL
Detection	wavelength	570 nm
Citric buffer sequence	pH	A: 6.00 B: 5.70 C: 5.45

Calibration curves were prepared in advance with biogenic amine chromatographic standards (see Fig. 1), based on means of triplicate data. Identification and quantification of each biogenic amine were accomplished by comparing retention time and peak area, respectively, between actual samples and standards.

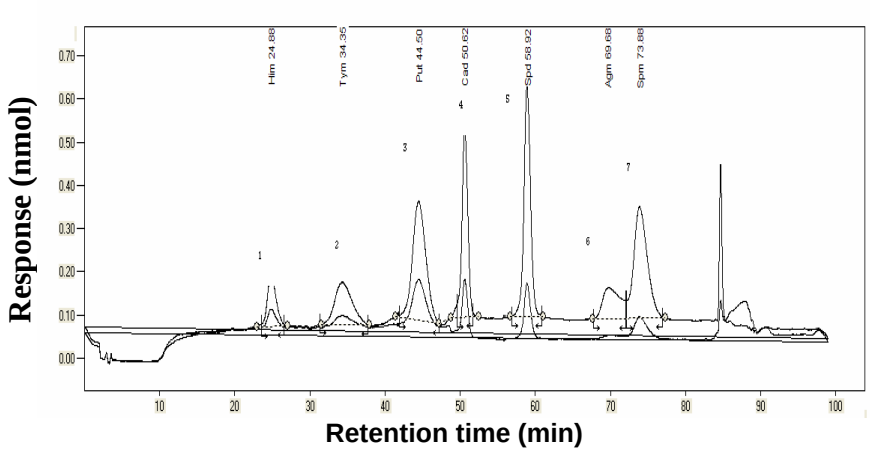

Fig 1: Amino acid analyzer chromatogram of biogenic amines standard
***1. Histamine 2. Tyramine 3. Putrescine 4. Cadaverine 5. Spermidine 6. Agmatine 7. Spermine**

Statistical analyses

Statistical analysis of the data produced was via one-way ANOVA and Tukey's test; statistical significance was declared at 5%.

RESULTS AND DISCUSSION

The average chemical composition of Edam cheese curd slurries containing probiotic bacterial strains, during ripening at 30°C, is presented in Table 2. Addition of probiotic bacterial strains apparently did not influence chemical composition. Similar results were reported by (Karakus *et al.,* 1995; Mara *et al.,* 1999; Ong *et al.* 2007).

Changes in pH, WSN, TCA-SN and PTA-SN will hereafter be taken as indices of ripening of Edam-type curd slurry. The pH of cheese curd slurries ranged from 5.60 to 5.15, and pH in slurries containing probiotic strains was slightly lower than that of the control. This could be explained by development of acidity at high incubation temperature (30ºC) when added with probiotic bacterial strains.

Table 2: Effect of combination of probiotic strains upon chemical composition of Edam cheese curd slurry during incubation.*

Item %	Incubation period (days)											
	7				14				21			
	C	P+B (1:1)	P+L (1:1)	P+B+L (1:1:1)	C	P+B (1:1)	P+L (1:1)	P+B+L (1:1:1)	C	P+B (1:1)	P+L (1:1)	P+B+L (1:1:1)
Total Solids	40.10	40.20	40.33	40.50	40.20	40.35	40.25	40.30	40.05	40.20	40.33	40.30
Salt	1.78	1.76	1.72	1.84	1.58	1.86	1.92	1.68	1.64	1.76	1.72	1.94
Fat	12.59	12.66	12.82	12.91	12.70	12.79	12.80	12.85	12.64	12.72	12.80	12.84

***C = Control Edam cheese curd containing L. lactis subsp. lactis**
P+B = Propionibacterium shermenii + Bifidobacterium bifidum (1:1)
P+L = Propionibacterium shermenii + Lactobacillus acidophilus (1:1)
P+B+L = Propionibacterium shermenii + Bifidobacterium bifidum + Lactobacillus acidophilus (1:1:1)

The curd slurry containing *B. bifidum* DSM 20082 + *L. acidophilus* ATCC4356 + *P. shermenii* PS-4 strains (1:1:1) exhibited the highest (*P<0.05*) concentration of WSN (Table 3). Kasimoglu *et al.,* (2004) reported that *L. acidophilus* produced an increase in this fraction during ripening of probiotic white cheese.

The levels of WSN, TCA-SN and PTA-SN increased in all samples during incubation as a consequence of proteolysis (Table 3) – but significant differences (*P<0.05*) between cheeses with and without added probiotic bacteria were noticeable.

Table 3:Effect of combinations of probiotic strains upon soluble nitrogen fractions of Edam cheese curd slurry during incubation.*

Nitrogen fractions (%) of TN	Incubation period (days)											
	7				14				21			
	C*	P+B (1:1)	P+L (1:1)	P+B+L (1:1:1)	C*	P+B (1:1)	P+L (1:1)	P+B+L (1:1:1)	C*	P+B (1:1)	P+L (1:1)	P+B+L (1:1:1)
WSN/TN**	5.40a	7.98b	9.8c	10.6c	7.75a	10.87b	12.5c	13.88d	10.89a	12.88b	14.76c	16.77d
NPN/TN**	3.15a	4.35b	4.99c	5.39d	4.10a	4.90b	5.88c	6.55d	5.87a	7.44b	7.34b	7.90b
AN/TN**	2.25a	3.66b	4.44c	5.27d	3.88a	5.66b	6.88c	7.86d	4.66a	5.44b	6.25c	7.88d
pH	5.60a	5.45a	5.25b	5.15b	5.60a	5.35b	5.30b	5.00c	4.70a	4.75a	4.90b	4.95b

***C = Control Edam cheese curd containing L. lactis subsp. lactis**
P+B = Propionibacterium shermenii + Bifidobacterium bifidum (1:1)
P+L = Propionibacterium shermenii + Lactobacillus acidophilus (1:1)
P+B+L = Propionibacterium shermenii + Bifidobacterium bifidum + Lactobacillus acidophilus (1:1:1

The value of WSN in the control increased 2.3 fold by the end of incubation, and the evolution in WSN, TCA-SN and PTA-SN was similar to

that reported by (Macedo *et al.,* 1997). The obtained results indicate that probiotic bacteria present a significant effect upon secondary proteolysis. In addition, Edam-type slurry inoculated with *B. bifidum* DSM 20082 + *L. acidophilus* ATCC4356 + *P. shermenii* PS-4 strains (1:1:1) showed the highest (*P<0.05*) concentration of WSN (16.8 %) and TCA-SN (7.8 %). These results resemble those of Madkor *et al.,* (2000), who reported that adjunct cultures of similar strains of lactobacilli can increase the level of PTA-SN. Whereas, Drake et al. (1996) found that *Lactobacillus helveticus*, used as adjunct starter in reduced fat Cheddar, also produces significantly greater rates of proteolysis than the control.

The results on flavour intensity in assessment between control and probiotic Edam cheese curd slurries are shown in Table 4.

Table 4: Effect of combinations of probiotic strains upon development of flavour intensity of Edam cheese curd slurry during incubation.*

Incubation period	Probiotic strains combinations											
	C	P+B (1:1)	P+L (1:1)	P+B+L (1:1:1)	C	P+B (1:1)	P+L (1:1)	P+B+L (1:1:1)	C	P+B (1:1)	P+L (1:1)	P+B+L (1:1:1)
7	4.40	4.65	4.85	4.90	4.95	5.35	5.5	5.88	5.30	5.60	5.80	5.88
14	4.75	4.85	4.99	5.10	5.1	5.9	5.88	5.55	5.60	5.44	5.90	5.90
21	4.75	5.50	5.74	5.87	5.20	5.30	5.45	5.65	5.65	5.74	5.85	5.92

***C = Control Edam cheese curd containing L. lactis subsp. lactis**
P+B = Propionibacterium shermenii + Bifidobacterium bifidum (1:1)
P+L = Propionibacterium shermenii + Lactobacillus acidophilus (1:1)
P+B+L = Propionibacterium shermenii + Bifidobacterium bifidum + Lactobacillus acidophilus (1:1:1)

Addition of P. shermenii + L. acidophilus (1:1) or P. shermenii + B. bifidum (1:1) or P. shermenii + L. acidophilus + B. bifidum (1:1:1) significantly improved the flavour of Edam cheese curd slurries; this could be attributed to the higher concentration of soluble nitrogenous compounds.

Five biogenic amines, viz. histamine, tyramine, putrescine, spermine and spermidine were found in Edam curds slurries; both control and experimental ones (see Fig 2, 3, and 4). The control Edam curd slurry (Fig 2.) showed the highest concentration of total BAs (478.84 mg/kg DW by 21 days), which almost doubled between 7 and 21 days.

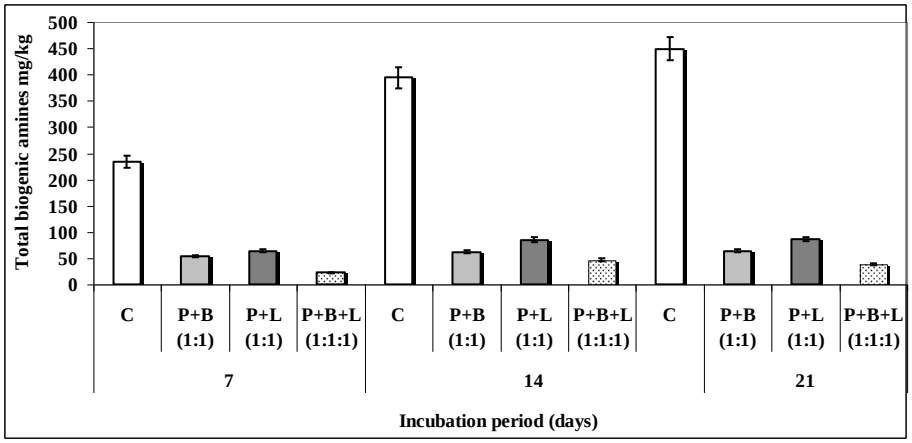

Fig. 2 Changes in the contents of total biogenic amines in Edam cheese curd slurry during incubation.

C = Control Edam cheese curd containing *L. lactis* subsp. *lactis
P+B = *Propionibacterium shermenii* + *Bifidobacterium bifidum* (1:1)
P+L = *Propionibacterium shermenii* + *Lactobacillus acidophilus* (1:1)
P+B+L = *Propionibacterium shermenii* + *Bifidobacterium bifidum* + *Lactobacillus acidophilus* (1:1:1)

The lowest content (5.2-35.1 mg/kg DW) of total BAs was observed in Edam-type curd slurry inoculated with *P. shermenii* + *B. bifidum* + *L. acidophilus* (1:1:1), followed closely (35.2-63.1 mg/kg DW) by *P. shermenii* + *B. bifidum* (1:1), and finally by *P. shermenii* + *L. acidophilus* (1:1) (46.8-74.84 mg/kg DW) as illustrated in Fig. 2.Similar results in terms of proteolytic breakdown were reported by Komprda *et al.,* (2007a) and Komprda *et al.,* (2008b). A rapid pH decrease may prevent BA formation in fermented products; and starter cultures able to outgrow nonstarter bacteria during late ripening and storage may also avoid excessive BA buildup (Suzzi *et al.,* 2003) – for instance, Roig-Sagués *et al.,* (1999) claimed that *Lactobacillus sake* is more suitable than *Lactobacillus curvatus* as starter culture if low BA levels is used as processing goal.

The changes in concentration of HIS are depicted in Fig. 3; this BA was found at high content 148.2 mg/kg Dw in non-inoculated Edam cheese – which is 3-fold the recommended maximum level (EFSA, 2011) by 21 days.

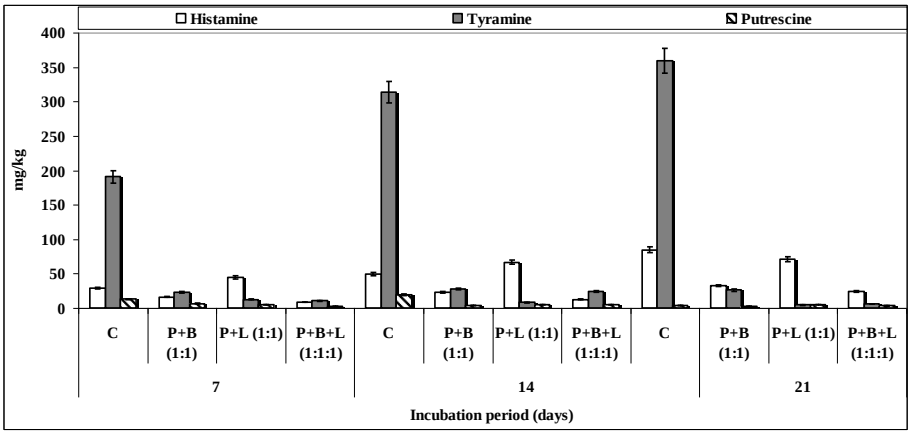

Fig. 3 Changes in the contents of histamine, tyramine and putrescine in Edam cheese curd slurry during incubation

C = Control Edam cheese curd containing *L. lactis* subsp. *lactis
P+B = *Propionibacterium shermenii* + *Bifidobacterium bifidum* (1:1)
P+L = *Propionibacterium shermenii* + *Lactobacillus acidophilus* (1:1)
P+B+L = *Propionibacterium shermenii* + *Bifidobacterium bifidum* + *Lactobacillus acidophilus* (1:1:1)

A reduction of HIS was observed with *P. shermenii* + *B. bifidum* + *L. acidophilus* (1:1:1); its concentration was lower than the control 238 and the other probiotic bacterial combinations tested.

The content of TYR remained high in non-inoculated cheese samples, and ranged from 190.4 to 359.2 mg/kg DW during the ripening period (see Fig. 3). On the other hand, addition of some strain combinations dramatically reduced formation of TYR during ripening: *P. shermenii* + *B. bifidum* + *L. acidophilus* (1:1:1) was more effective in this respect. Priyadarshani et al. (2011) showed that BA reduction was not apparent for *L. acidophilus*, *L. lactis* subsp. *lactis*, *L. lactis* subsp. *lactis* and *Lactobacillus plantarum*; they claimed that BA formation is strain dependent but not related only to species. Therefore, careful screening for amino acid decarboxylase activity is recommended before selecting LAB or probiotic strains as starter in dairy industry (Komprda et al 2007a; Komprda et al 2008b; Priyadarshani *et al.,* 2011). Similar results were obtained by (Bunková *et al.,* 2010) for selected biogenic amines (HIS, TYR, and PUT) in 4 layers of Dutch-type cheese (Edam-cheese), depending on the ripening/ storage regime followed along cheese ripening.

The combination consisting of *P. shermenii* + *B. bifidum* + *L. acidophilus* (1:1:1) was more pronounced in the reduction of PUT; it increased from 12.8 mg/kg DW by 7 days to a 2.6-fold value by 21 days of ripening (see Fig. 3). This result in a dairy matrix is consistent with the reduction in putrescine in sauerkraut (plant matrix) inoculated with *Lactobacillus plantarum*, *L. casei* and *Lactobacillus curvatus* (Rabie *et al.,* 2011a) and in sukuc (meat matrix) inoculated with *Lactobacillus sakei* and *Staphylococcus carnosus* (Gençcelep *et al.,* 2007) – so acidity may play a

role. Finally, the maximum contents of spermidine and spermine in non-inoculated Edam cheese were 3.1 and 3.6 mg/kg DW, as shown in Fig. 4. There is also a decrease of 1.6 and 2.1-fold of the concentration of spermidine and spermine, especially in the case of Edam cheese inoculated with *P. shermenii* + *B. bifidum* + *L. acidophilus* (1:1:1). These two amines accounted for the lowest concentrations.

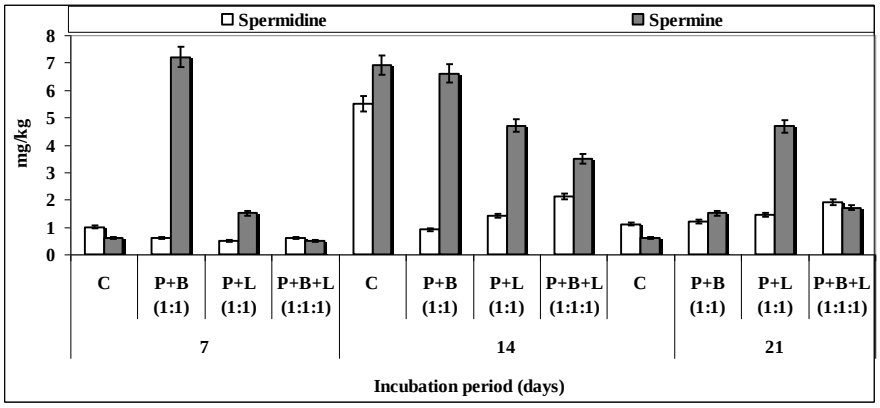

Fig. 4 Changes in the contents of spermidine and spermine in Edam cheese curd slurry during incubation.

C = Control Edam cheese curd containing *L. lactis* subsp. *lactis
P+B = *Propionibacterium shermenii* + *Bifidobacterium bifidum* (1:1)
P+L = *Propionibacterium shermenii* + *Lactobacillus acidophilus* (1:1)
P+B+L = *Propionibacterium shermenii* + *Bifidobacterium bifidum* + *Lactobacillus acidophilus* (1:1:1)

The unique performance of the co-cultures added containing the two probiotic strains (i.e. *B. bifidum* and *L. acidophilus*) arises likely from their absence of the enzymatic machinery required to decarboxylate free amino acids, coupled with a putative capacity to actually take up existing BAs; their ecological dominance in the curd slurry will eventually hamper net formation of biogenic amines, and even actively contribute to reduce the current level of BAs.

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التحلل البروتينى وتكوين الامينات الحيوية فى معلق جبن الايدم المعقمة و الملقحة ببعض التوليفات من سلالات البكترية الحيوية

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في هذا البحث تم تلقيح معلق الخثرة المعقمة لجبن الايدام (المقارنة) بنسبة ١٪ من بكتريا ***Lactococcus lactis subsp. lactis*** KF147 ولدراسة تأثير اضافة بعض التوليفات من سلالات البكتري الحيوية على التحلل البروتينى وتكوين الأمينات الحيوية اثناء التحضين على درجة ٣٠م لمدة ٢١ يوم فقد تم تلقيح معلق الخثرات المعقمة لجبن الادام بنسبة ١% من التوليفات الأتية: ***Propionibacterium shermenii*** PS-4 + ***Bifidobacterium bifidum*** DSM 20082 (1:1), ***Propionibacterium shermenii*** PS-4 **+** ***Lactobacillus acidophilus*** (1:1) ATCC4356, or ***Propionibacterium*** + ***shermenii*** PS-4 + ***Bifidobacterium bifidum*** DSM 20082 ***Lactobacillus acidophilus*** ATCC4356 (1:1:1). وأظهرت النتائج عدم وجود تأثير معنوى من أي توليفة من البكتريا الحيوية على المواد الصلبة الكلية والملح والدهن لمعلق الخثرات المعقمة لجبن الايدام. ومع ذلك، فقد وجد ان تلقيح الخثرات المعقمة لجبن الايدام قد أظهر تاثيرا على رقم الحموضة والمركبات النيتروجينية الذائبة كما أظهرت التوليفة التى تتكون من ***Propionibacterium shermenii*** + ***Bifidobacterium bifidum*** + ***Lactobacillus acidophilus*** (1:1:1) أعلى تركيز (٧.٩٪) من النيتروجين الذائب في الماء في نهاية فترة التحضين. وعندما اشتمل التلقيح على ***Bifidobacterium bifidum*** لوحظ انخفاض كبير في تركيز الأمينات الحيوية الكلية (٤٤٧-٣٧ ملليجرام / كجم بالنسبة للوزن الجاف) كمالوحظ ايضا انخفاض فى تركيز الهستامين (٨٤-٢٥) والتيرامين (٣٥٩ - ٦ ملليجرام / كجم بالنسبة للوزن الجاف) على التوالى.