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PROTEOLYSIS AND BIOGENIC AMINE FORMATION IN STERILIZED EDAM-TYPE CURD SLURRY INOCULATED WITH PROBIOTIC STRAINS

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ABSTRACT

Edam-type curd slurry inoculated with 1% *Lactococcus lactis* subsp. *lactis* KF147 as control and further added with *Propionibacterium shermanii* PS-4 +*Bifidobacterium bifidum* DSM 20082 (1:1), *P. shermanii* PS-4 +*Lactobacillus acidophilus* ATCC4356 or *P. shermanii* PS-4 +*B. bifidum* DSM 20082 +*Lacto- bacillus acidophilus* ATCC4356 (1:1:1), at a rate of 1%, were studied for their effect upon biogenic amine and proteolysis pattern during incubation at 30C for 21 days. Results showed no significant influence of any combination of probiotic microorganisms on total solids, salt and fat of Edam-type curd slurries, but some effect on pH and soluble nitrogen fractions; presence of *P. shermanii* + *B. bifidum* + *Lactobacillus acidophilus* led to the highest concentration (7.9%) of water-soluble nitrogen by the end of incubation. When inoculation included *B. bifidum*, a significant decrease in total biogenic amines (from 447 to 37 mg/ kgDW) was observed by 21 days – with histamine decreasing from 84 to 25 and tyramine from 359 to 6 mg/kgDW.

PRACTICAL APPLICATIONS

This study was aimed at investigating proteolysis and biogenic amine formation by selected probiotic bacteria added to Edam-type curd slurry. The nitrogen fractions and biogenic amines in control increased significantly throughout incubation at 30C. Inocula of *P. shermanii +L. acidophilus +B. bifidum* (1:1:1) could effectively reduce biogenic amine contents, especially histamine and tyramine. This observation has potential public health impact because regular curds are often above the maximum legal threshold in regions with warm weather and poor cold storage network. Furthermore, addition of probiotic *L. acidophilus* and *B. bifidum* improved the overall flavor profile, owing to extra soluble nitrogen produced.

INTRODUCTION

During cheese ripening, several different biochemical changes occur: flavor and texture characteristic of each cheese variety develop along aging, via transformation of protein, fat and residual lactose to primary products that are further degraded to secondary products (Kheadr *et al*. 2003; McSweeney 2004). Proteolysis is a major event in cheese ripening; the proteolytic system of the starter contributes strongly thereto during ripening (Fox *et al*. 1993;

Lane and Fox 1996; Tungjaroenkai *et al*. 2001).

Starter cultures account for a major portion of the micro- flora in young cheese curd; during ripening, starter numbers decrease owing to cell death and subsequent autolysis (Crow *et al*. 1995). Intracellular peptidases released by autolysis can hydrolyze casein to smaller peptides and eventually amino acids, and further microbial action then transforms them to amines, alcohols and sulphur- containing compounds that play an important role in flavor formation (Cogan and Beresford 2002). Comprehensive, experimental evidence suggests that adjunct cultures or nonstarter lactic bacteria also play a role in cheese ripening (Peterson and Marshall 1990; Rodriguez 1998). Considering the extended survival and viability of probiotic cultures, cheese has been recommended as a better carrier of probiotic strains than other fermented milk products. Owing to its solid consistency that offers greater protection along the gastrointestinal tract (Stratton *et al*. 1991; Ong *et al*. 2006), this has been consubstantiate for probiotic species such 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*. 1999) – whereas Vinderola *et al*. (2002) emphasized the interactions between mesophilic starter and probiotic cultures in cheese.

Biogenic amines (BAs) in fermented food (including cheese) have a high impact on public health, as they are potentially toxic to humans at levels above 750–900 mg/kg (Ten-Brink *et al*. 1990; Spanjer and van Roode 1991). BAs are low-molecular nitrogenous compounds formed chiefly via microbial decarboxylation of precursor amino acids (Halász *et al*. 1994), so they can serve as an indicator of food quality (Rauscher-Gabernig *et al*. 2009). European Food Safety Authority has recently set the maximum daily intake of histamine to 50 mg and tyramine to 600 mg for healthy individuals, although such limits may be drastically reduced in the case of reported intolerance or use of monoamine oxidase inhibitor drugs (European Food Safety Authority and Panel on Biological Hazards (BIOHAZ) 2011). Thanks to the oxidation system encompassing monoamine and diamine oxidases, small amounts of BAs can be metabolized in the organism without a major impact on health (Rice *et al*. 1976; Taylor 1986), but ingestion of higher levels brings about serious conditions, e.g., release of adrenaline and noradrenaline leading to gastric acid secretion, increased cardiac output, migraine, tachycardia and high blood pressure (Shalaby 1996; Loret *et al*. 2005); furthermore, a fraction of the population has histamine (HIS) intolerance (Maintz and Novak 2007).

The ability to decarboxylate amino acids is present in many genera of lactic acid bacteria (LAB) (Halász *et al*. 1994; Arena and Manca de Nadra 2001) – and tyramine (TYR), HIS, putrescine (PUT) and cadaverine can actually be found to considerable levels and other BAs to lesser amounts (Spano *et al*. 2010). A few strains of LAB, e.g., *Lactococcus lactis* subsp. *lactis*, have exhibited decarboxylase activity (Lorencová *et al*. 2012); however, lactobacilli such as *Lactobacillus hilgardii* and *Pediococcus parvulus* have also been associated with the release of HIS (Lucas *et al*. 2005; Moreno-Arribas and Polo 2008) and *enterococci* with the release of TYR (Bover-Cid and Holzapfel 1999). PUT synthesis was initially associated to gram-negative bacteria, particularly members of Enterobacteriaceae (Chaves-López *et al*. 2006). The pH optima 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 2004) and *Lactococcus lactis* is presumably responsible for the appearance of BAs in cheese where it has been used as starter culture.

This work intends to fill the gap of knowledge on Edam- type curd slurries, encompassing especially BA release, by ascertaining the effects of adding probiotic strains to the regular

inocula of LAB.

MATERIALS AND METHODS

Cow's Milk

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

Starter and Probiotic Microorganisms

Lactococcus lactis subsp. *lactis* KF147 and *Propionibacterium shermanii* PS-4 were obtained from Chr. Hansen Laboratories (Copenhagen, Denmark). *B. bifidum* DSM 20082 and *Lactobacillus acidophilus* ATCC4356 were obtained from Cairo Microbiological Center, MIRCEN, Faculty of Agriculture, Ain Shams University (Cairo, Egypt). Stock cultures were kept frozen at −70C in 30% v/v glycerol. Prior to use, each strain was cultivated in de Man, Rogosa & Sharpe (MRS) broth for 48 h and underwent two consecutive transfers. When used as adjuncts, the aforementioned strains were inoculated at 0.1% into sterile 3% fat milk and incubated for 24 h at the appropriate temperature.

Preparation of Edam-Type Curd Slurry

Cow's milk was pasteurized at 72C for 15 s, cooled to 32C and transferred to a sterile cheese vat under a laminar flow hood. Approximately 100 L of milk was used to manufacture Edam-type curd, following procedures described by Scott (1981).

Cheese curd slurry was prepared following Farkye *et al*. (1995); 100 g of cheese curd was added with 2 g of NaCl and stirred for a few minutes to assure uniformity in salt profile and transferred aseptically into a sterile, wide mouth bottle where it underwent sterilization at 121C for 15 min. All sterilized curd slurries were aseptically inoculated with 1% of Edam cheese starter. One part of curd slurry without probiotic stains served as control. The other portions of curd slurry were aseptically inoculated with a combination of *Pr. shermanii* PS-4 +*B. bifidum* DSM 20082, *Pr. shermanii* PS-4 + *Lactobacillus acidophilus* or *Pr. shermanii* PS-4 +*Lactobacillus acidophilus* ATCC4356 + *B. bifidum* DSM 20082 (1:1:1) at a rate of 1%. Each curd slurry preparation was replicated three times using freshly made starter cheese curd. Curd slurry bottles were incubated at 30C for 7, 14 and 21 days.

Assessment of Proteolysis

The pH 4.6 water-soluble nitrogen (WSN) as well as the nonprotein nitrogen (NPN; or nitrogen soluble in 12% trichloroacetic acid) and the amino acid nitrogen (AN; or nitrogen soluble in phosphotungstic acid) all referred to total nitrogen (TN) and were determined as described by Gripon *et al*. (1975).

Extraction of Bas

BAs in curd slurry samples were extracted as originally described by Simon-Sarkadi and Holzapfel (1994) and later improved by Rabie *et al*. (2011a) for cheese. To extract BAs, 10 mL of 10% trichloroacetic acid was added to 3-g samples of cheese and the mixture was shaken for 1 h using a Laboshake LS 500i (Gerhardt, Germany). The extract was filtered through Whatman No.1 filter paper (Sigma-Aldrich, St. Louis, MO). To remove the fat content, the samples were kept at −20C for 1 day and then centrifuged (MLW, T 24, Leipszig,

Germany) at 7,000× *g* for 15 min. Supernatants were collected and filtered through a 0.25-μm membrane filter (Nalgene, Rochester, NY)

Quantification of Bas

The analysis of BAs was performed with an AAA 400 amino acid analyser (Ingos, Prague, Czech Republic), equipped with an Ostion LG ANB ion-exchange column (Lachema, Czech Republic). Colorimetric detection was accomplished at 570 and 440 nm, after post-column derivatization with ninhydrin (Csomos and Simon-Sarkadi 2002). Details of the analytical procedure are available elsewhere (Rabie *et al*. 2011c).

All analyses were performed in duplicate and their values (average ± standard deviation) are made available in Tables 1 and 2

Statistical Analyses

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

RESULTS AND DISCUSSION

Changes Soluble Nitrogenous Compounds

Total solids, salt, fat and pH exhibited no significant differences between types of cheese, except with regard to pH. Changes in WSN/TN, NPN/TN and AN/TN will hereafter be taken as indices of ripening of Edam-type curd slurry. The curd slurry containing *B. bifidum* DSM 20082 + *Lactobacillus acidophilus* ATCC4356+*Pr. shermanii* PS-4 strains (1:1:1) exhibited the highest ($P < 0.05$) concentration of WSN/TN (Table 1). Kasimoglu *et al*. (2004) reported that *Lactobacillus acidophilus* produced an increase in this fraction during ripening of probiotic white cheese.

The levels of WSN/TN, NPN/TN and AN/TN increased in all samples during incubation as a consequence of proteolysis (Table 1), but significant differences ($P < 0.05$) between types of cheese with and without added probiotic bacteria were noticeable. The value of WSN/TN in the control increased by 2.3-fold at the end of incubation and the evolution in WSN/TN, NPN/TN and AN/TN was similar to that reported by Macedo and Malcata (1997). Our results indicate that probiotic bacteria do have a significant effect upon secondary proteolysis. In addition, Edam-type slurry inoculated with *B. bifidum* DSM 20082 + *Lactobacillus acidophilus* ATCC4356 + *Pr. shermanii* PS-4 strains (1:1:1) showed the highest ($P < 0.05$) concentration of WSN/TN (16.8%) and NPN/TN (7.8%). These results resemble those of Madkor *et al*. (2000), who concluded that adjunct cultures of similar strains of lactobacilli can increase the level of NPN/TN; 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.

Bas

Five BAs, viz. HIS, TYR, PUT, spermine and spermidine were found in Edam-type curds, both control and experimental ones (see Table 2). The control Edam-type curd slurry (Table 2) showed the highest concentration of total BAs (478.84 mg/kgDW by 21 days), which almost doubled between 7 and 21 days.

The lowest content (5.2–35.1 mg/kgDW) of total BAs was observed in Edam-type curd slurry inoculated with *Pr. shermanii* + *B. bifidum* +*Lactobacillus acidophilus* (1:1:1), followed closely (35.2–63.1 mg/ kgDW) by *Pr. shermanii* + *B. bifidum* (1:1) and finally by *Pr. shermanii* + *Lactobacillus acidophilus* (1:1) (46.8–74.84 mg/kgDW) as illustrated in Table 2. Similar results in terms of proteolytic breakdown were reported by Komprda *et al.* (2007, 2008). Remember that enzymatic decarboxylation of an amino acid leads to the corresponding BA (i.e., TYR and HIS result from decarboxylation of TYR and HIS, respectively), besides the release of carbon dioxide (Joosten and Stadhouders 1987). The different profile observed is likely the result of the distinct enzymatic machinery of *B. bifidum* that overrides the effect of *Pr. shermanii* and *Lactobacillus acidophilus*. Despite HIS and TYR being traditionally associated with Edam-type curds (McSweeney and Sousa 2000), their sensory effect is marginal, so the final typical flavor will not be compromised by the experimental inocula tested. The effect upon BAs may be enhanced by a rapid pH decrease that is compatible with *B. bifidum* viability. Starter cultures able to outgrow nonstarter bacteria during late ripening and storage have been proven to avoid excessive BA build-up (Suzzi and Gardini 2003); for instance, Roig-Sagués and Eerole (1999) claimed that *Lacto- bacillus sake* is more suitable than *Lactobacillus curvatus* as starter culture if low BA levels are used as processing goal.

Further discussion focuses chiefly on 21-day curd slurries because this is the maximum expected period of incubation prior to consumption.

The changes in concentration of HIS are depicted in Table 2. This BA was found at high content of 148.2 mg/ kgDW in control Edam-type curd, which is threefold the recommended maximum level (European Food Safety Authority and Panel on Biological Hazards (BIOHAZ) 2011) by 21 days. A reduction of HIS was observed with *Pr. shermanii* +*B. bifidum* +*Lactobacillus acidophilus* (1:1:1); its concentration was lower than the control and the other probiotic bacterial combinations tested. Hence, the role of probiotic strains is emphasized when two types thereof are added.

The content of TYR remained high in control curd slurry samples and ranged from 190.4 to 359.2 mg/kgDW during the incubation period (see Table 2). On the other hand, addition of some strain combinations dramatically reduced the formation of TYR during incubation: *Pr. shermanii* + *B. bifidum* +*Lactbacillus acidophilus* (1:1:1) was more effective in this respect, once again confirming the results pertaining to HIS. Priyadarshani and Rakshit (2011) showed that BA reduction was not apparent for *Lactobacillus acidophilus*, *Lactobacillus 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*. 2007, 2008; Priyadarshani and Rakshit 2011). Similar results were obtained by Bunková *et al*. (2010) for selected BAs (HIS, TYR and PUT) in four layers of Dutch-type cheese (Edam cheese), depending on the ripening/storage regime followed along cheese ripening.

The combination consisting of *Pr. shermanii* + *B. bifidum* +*Lactobacillus acidophilus* (1:1:1) was once again the most pronounced toward reduction of PUT; it decreased from 12.8 mg/kgDW by 7 days to approximately one-fourth by 21 days of incubation (see Table 2). This result in a dairy matrix is consistent with the reduction in PUT in sauerkraut (plant matrix) inoculated with *Lactobacillus plantarum*, *Lactobacillus casei* and *Lactobacillus curvatus* (Rabie *et al*. 2011b), and in sukuc (meat matrix) inoculated with *Lactobacillus sakei*

and *Staphylococcus carnosus* (Gençcelep *et al*. 2007) – so acidity may play a role.

The maximum contents of spermidine and spermine in control Edam-type curd were 3.1 and 3.6 mg/kgDW, respectively, as shown in Table 2. There was a decrease of 1.6 and 2.1-fold mg/kgDW of their concentration in the case of Edam-type curd inoculated with both probiotics, i.e., *Pr. shermanii+B. bifidum+Lactobacillus acidophilus* (1:1:1). These two amines accounted for the lowest concentrations detected in both control and experimental probiotic cheese curd slurries.

The unique performance of the co-cultures added containing the two probiotic strains (i.e., *B. bifidum* and *Lacto- bacillus 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 BAs and even actively contribute to reduce the current level of BAs. On the other hand, pH decreases have been implicated in low rates of release of BAs in meats – and our probiotic cultures were effective acidifiers. In our case, *Lactobacillus lactis* subsp. *lactis* should account for any BA detected in controls, owing to the defined cultures used and its presence in all experimental Edam-type curd slurries.

CONCLUSION

The contents of soluble nitrogenous compounds and BAs in control and experimental Edam-type curd slurry increase significantly throughout incubation at 30C. However, the co-inocula *Pr. shermanii +Lactobacillus acidophilus + B. bifidum* (1:1:1) can effectively reduce BA contents – from 479 in control down to 35 mg/kgDW; an emphasis exists on HIS, corresponding to approximately 16-fold decrease – rel- evant for qualitative reasons as per its toxicity, and also for tyramine – relevant for quantitative reasons owing to its level. This might convey a favorable contribution to public health in regions characterized by warm temperatures and poor cold storage network, where ripened semihard cheese is frequently included in the diet. On the other hand, addition of those two probiotic strains is expected to improve the overall flavor profile because of the presence of extra soluble nitrogen.

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TABLE 1. EFFECT OF COMBINATIONS OF PROBIOTIC STRAINS UPON pH AND SOLUBLE NITROGEN FRACTIONS OF EDAM-TYPE CURD SLURRY DURING RIPENING

Nitrogen fractions (%)	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

For each incubation period and for each nitrogen type, figures followed by different letters indicate statistically significant ($P < 0.05$) differences between inoculated cultures.
AN/TN, ratio of amino acid nitrogen to total nitrogen; C, control Edam-type curd containing *Lactobacillus lactis* subsp. *lactis*; NPN/TN, ratio of non-protein nitrogen to total nitrogen; P + B, *Propionibacterium shermanii + Bifidobacterium bifidum*; P + B + L, *Propionibacterium shermanii + Bifidobacterium bifidum + Lactobacillus acidophilus*; P + L, *Propionibacterium shermanii + Lactobacillus acidophilus*; WSN/TN, ratio of water-soluble nitrogen to total nitrogen.

TABLE 2. EFFECT OF COMBINATIONS OF PROBIOTIC STRAINS UPON BIOGENIC AMINES OF EDAM-TYPE CURD SLURRY DURING RIPENING

Biogenic amines (mg/kg)	Ripening 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)
Histamine	28.7b	16.1c	44.4a	8.4c	49.3b	23.2c	66.4a	12.6c	84.1b	32.6d	71.0a	24.5a
Tyramine	190.4a	23.3b	12.3c	10.4d	313.3a	27.8b	7.9c	23.8c	359.2a	25.9b	4.33b	6.0b
Putrescine	12.8a	6.4b	5.1c	2.3c	19.1a	3.6b	4.5b	4.7b	3.9a	2.6b	4.49a	3.1a
Spermidine	1.0a	0.6a	0.5a	0.6a	5.5a	0.9c	1.4bc	2.1b	1.1a	1.2a	1.45a	1.9a
Spermine	0.6b	7.2a	1.5b	0.5b	6.9a	6.6a	4.7b	3.5b	0.6a	1.5b	4.68c	1.7b
Total	233.5a	53.6b	63.8b	22.3c	394.1a	62.1c	84.9b	46.7d	448.9a	63.8b	85.95b	37.43b

For each incubation period and for each biogenic amine, figures followed by different letters indicate statistically significant ($P < 0.05$) differences between inoculated cultures.
C, control Edam-type curd containing *Lactobacillus lactis* subsp. *lactis*; P + B, *Propionibacterium shermanii + Bifidobacterium bifidum*; P + B + L, *Propionibacterium shermanii + Bifidobacterium bifidum + Lactobacillus Acidophilus*; P + L, *Propionibacterium shermanii + Lactobacillus acidophilus.*