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Isolation, Technological Characterization and Safety Assessment of Potential Adjunct Cultures of Lactic Acid Bacteria

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ABSTRACT

Adjunct cultures are non-starter microorganisms contributing to the development of favorable flavor and texture during cheese ripening. The present study was designed to isolate and characterize potential adjunct cultures of lactic acid bacteria (LAB) from pickled Domiatti cheese and Ras cheese. Fifty-four cheese samples including 33 and 21 samples of pickled Domiatti cheese and Ras cheese, respectively were randomly collected from Mansoura city and villages in its vicinity and assessed for their flavor by panelists. Given the association of the adjunct cultures with typical flavor in cheese, cheese samples with "good" or "acceptable" flavor were further analyzed as a potential source of adjunct LAB cultures. A total of 162 suspected LAB isolates could be recovered from theses samples, of which 37 and 4 isolates were confirmed to belong to the *Enterococcus* and *Lactobacillus* genera, respectively. Further biochemical identification to the species level showed that the *Enterococcus* isolates involved *Ent. faecalis* (2 isolates), *Ent. faecium* (7 isolates), *Ent. gallinarum* (2 isolates), *Ent. durans* (1 isolate), *Ent. mundtii* (2 isolates), *Ent. casseiliflavus* (2 isolates), *Ent. pseudoaavium* (12 isolates), and *Enterococcus* spp. (9 isolates). Whereas, *Lactobacillus* isolates could be identified as *Lb. plantarum* (1 isolate) and *Lactobacillus* spp. (3 isolates). *Enterococcus* and *Lactobacillus* isolates were examined for technological properties including acidity development, proteolytic activity, and lipolytic activity. They showed phenotypic diversity in those technological characteristics. Isolates were also assessed for safety-associated traits including antibiotic resistance, biogenic amine production, and hemolytic activity. *Enterococcus* isolates showed resistance to several antibiotics and were able to produce the biogenic amines histamine and tyramine. Hemolytic activity could be also detected in those isolates. *Lactobacillus* cultures showed resistance to only 2 out of 9 antibiotics and were unable to produce histamine or tyramine. They did not also show hemolytic activity. This study presents a collection of *Enterococcus* and *Lactobacillus* isolates to be assessed for use as adjunct cultures based on their technological and safety-related traits.

Keywords: Adjunct cultures, *Enterococcus*, *Lactobacillus*, proteolytic activity, lipolytic activity, antibiotic resistance, biogenic amine, hemolytic activity.

INTRODUCTION

Modern cheese making involves milk pasteurization, use of dairy starters, and the application of hygienic practices. While the use of pasteurization and hygienic practices ensure the safety of cheese, more favorable flavor and texture have been noted in cheese prepared from unpasteurized milk under artisanal conditions (El-Soda *et al*., 2000). This is due to the activity of non-starter microorganisms in raw milk and processing environment that could be eliminated by pasteurization and hygienic practices.

Non-starter microorganisms have been thus the subject of several studies aiming to improve the quality of cheese made from pasteurized milk by incorporating well-characterized and selected strains of those microorganisms in the form of adjunct cultures (Bintsis *et al.*, 2000; Kondyli *et al*., 2002; Katsiari *et al*., 2002; Awad *et al*., 2007; Awad *et al*., 2010). The effect of adjunct cultures on cheese qualities was found not to be only restricted to improving sensory traits but may also extent to biopreservation and health benefits of cheese (Awad *et al*., 2007; Dabiza and El-Deib, 2007; Nascimento *et al.*, 2008; Aljewicz *et al*., 2009; Vinderola *et al.*, 2009; Awad *et al*., 2010; Kumar *et al.*, 2015).

While there have been several studies addressing the effect of adjunct cultures on the quality of Western cheese varieties, relatively limited relevant research has been conducted in Egypt (Awad *et al*., 2007; Awad *et al*., 2010). The present study was thus designed to isolate potential adjunct cultures of lactic acid bacteria from popular Egyptian ripened cheese varieties. It also involved technological characterization and safety assessment of the isolates.

MATERIALS AND METHODS

Collection of cheese samples

A total of 54 Domiatti and Ras cheese samples were randomly collected from local markets in Mansoura city and villages in its vicinity. These specimens included 33 samples of pickled Domiatti cheese and 21 samples of Ras cheese. Samples were aseptically collected and preserved under refrigeration till analysis.

Sensory evaluation

All cheese samples were assessed for their characteristic typical flavor as described by Awad *et al*. (2010). A total score of 100 was used to assess the acceptability of samples that were graded as follows: 0-25, unacceptable; 26-50, poor; 51-75, acceptable; 76-100, good.

Isolation and identification of lactic acid bacteria (LAB)

Ten grams of each cheese sample were aseptically ground with 90 ml of sterile saline solution (0.85% NaCl). Aliquots of 100 µl of this suspension were platted onto kanamycin aesculine azide (KAA) agar (Oxoid, Basingstoke, UK) and M17 agar (Oxoid, Basingstoke, UK) for the isolation of enterococci and lactococci respectively, and Rogosa agar (Oxoid, Basingstoke, UK) for the isolation of lactobacilli. Plates were incubated for 24 h at 37 °C for KAA agar and Rogosa agar and 30 °C for M17 agar. White or grey colonies surrounded by black zones were picked up from KAA agar, while small white colonies were picked up from M17 agar, and Rogosa agar.

Suspected cocci and lactobacilli isolates of LAB were grown at 37 ºC for 24 h on tryptone soya agar (TSA) (Oxoid, Basingstoke, UK), and M17 agar, respectively. They were then subjected to Gram-staining

(Pollack *et al*., 2005) and the catalase test (Macfaddin, 1977). They were also examined for their ability to coagulate milk, and grow at 45 ºC, 10 ºC, pH 9.6 (Sharp, 1979), and in the presence of 4% and 6.5% NaCl (Abd El-Malek and Gibson,1948). Isolates were also tested for growth on the bile esculine agar (BEA) (Oxoid, Basingstoke, UK) and the production of CO_2 from glucose (De Man *et al*., 1960).

Confirmed *Enterococcus* and *Lactobacillus* isolates were further identified to the species level. They were grown in MRS broth at 37 °C for 24 h and examined for carbohydrate fermentation (Facklam, 1972) , pyruvate utilization (Gross *et al*., 1975), motility and hydrogen sulfide (H_2S) production (Harrigan and McCance, 1966) and pigment production (Facklam and Collins, 1989).

Assessment of technological properties of LAB isolates

Enterococcus and *Lactobacillus* isolates from Egyptian cheese samples were grown in tryptone soya broth (TSB) (Oxoid, Basingstoke, UK) and MRS broth, respectively at 37 °C for 24 h before every experiment. Isolates were examined for acidity development in sterilized reconstituted skim milk (RSM) (10% total solids) at 37 °C for 24 h.

Isolates were also screened for their proteolytic and lipolytic activities as described by Chalmers (1962) as follows. For proteolytic activity, aliquots of 100 µl from suitable serial dilutions of a 24 h culture of each isolate were plated onto TSA and M17 agar supplemented with 1% sterilized RSM (10% total solids) for enterococci and lactobacilli, respectively. Plates were incubated at 37 °C for 24 h and observed for clear zone formation after the addition of HCl (0.1 N) on the medium surface. The same protocol was also used for examining lipolytic activity by adding 0.4% fat from cream to the platting medium rather than sterilized RSM. Plates were observed for blue-green colonies following the addition of $CuSO_4$ solution (20%).

Proteolytic activity of LAB isolates was quantified by using the colorimetric method of Hull (1947), modified by Citti *et al*. (1963) as follows. Aliquots of 5 ml of 24 h cultures were washed and re-suspended in 0.32 mM sodium phosphate buffer (pH 7), and inoculated at 1% (v/v) into UHT skim milk (8.5% total solids), followed by incubation at 37 °C for 24 h. Then, Trichloroacetic acid solution (TCA) (0.72 N) was added and the mixture was held for 10 min at room temperature, followed by centrifugation (5000 rpm /10 min). The soluble protein in the supernatant was determined as described by Lowry *et al.* (1951). One milliliter of the resultant supernatant was mixed well with 5 ml of reagent C prepared by mixing 50 ml of reagent A (Na_2CO_3, 2% + NaOH, 0.4%) with 1 ml of reagent B ($CuSO_4.5H_2O$, 0.5% + potassium or sodium tartrate, 1%). This was followed by incubation for 10 min at room temperature. An amount of 0.5 ml of diluted folin-Ciocalteu phenol reagent (1:1) was then added and mixed well, followed by incubation for 30 min at room temperature, which resulted in the development of blue color. The intensity of this color was assessed by measuring light absorbance (A) at a wave length of 650 nm. Soluble protein concentration was calculated by plugging the A_{650} reading into the line equation of a standard tyrosine curve that related the concentration of tyrosine to A_{650}. The standard tyrosine curve was produced by mixing different tyrosine concentrations with reagent C followed by the same steps described above.

Lipolytic activity of LAB isolates was also quantified by examining their ability to produce volatile fatty free acids from milk fat as follows. A 24 h culture of each isolate was inoculated at 1% (v/v) into UHT full milk (3% fat, and 8.25% total solids), followed by incubation at 37 °C for 24 h. Then, the total volatile fatty free acids (TVFFA) was determined as described by Kosikowski (1978). Aliquots of 2.5 g from each sample were digested with 8.5 g $MgSo_4$, 10 ml H_2So_4 (10%), and 62.5 ml distilled water, followed by distillation. A distillate amount of 75 ml was collected and titrated by using NaOH (1/10 N) and phenolphthaline as indicator. Results were expressed as volume of NaOH (0.1 N) used in the titration of TVFFA in 100 g sample.

Assessment of antibiotic resistance

The antibiotic sensitivity of LAB isolates was assessed using the Kirby-Bauer disc-diffusion method (Fortina *et al*., 2008). This involved 9 antibiotics: ampicillin (10 µg), penicillin (10 µg), clindamycin (2 µg), gentamycin (10 µg), kanamycin (10 µg), streptomycin (10 µg), lincomycin (10 µg), chloramphenicl (30 µg), and oxacillin (1 µg). Results were interpreted according to the criteria of the Clinical and Laboratory Standards Institute (CLSI) (2011).

Screening of biogenic amine production

LAB isolates were screened for their ability to produce the biogenic amines histamine and tyramine using the method described by Bover-Cid and Holzapfel (1999).To induce the expression of the decarboxylase enzymes, each LAB isolate was grown at 37 ºC for 24 h in TSB for enterococci, and MRS broth for lactobacilli supplemented with 0.1% (w/v) histidine or tyrosine. Cultures were then serially diluted and platted onto TSA or M17 agar, for enterococci and lactobacilli, respectively. Both TSA and M17 were supplemented with 1% (w/v) histidine or tyrosine and 0.06% bromocresol purple. Plates were incubated at 37 ºC for 24 h. The appearance of purple colonies indicated the production of biogenic amines from histidine or tyrosine. The disappearance of tyrosine precipitates around the colonies was also taken as an additional indicator of a positive result.

Hemolytic activity

The hemolytic activity of LAB isolates was determined using the method described by De Vuyst *et al.* (2003). Serial dilutions of a 24 h culture of *Enterococcus* and *Lactobacillus* isolates were platted onto TSA and M17 agar supplemented with 5% (v/v) horse blood, respectively. This was followed by incubation at 37 ºC for 24 h. Positive hemolytic reactions were recorded when clear zones appeared around the colonies.

RESULTS AND DISCUSSION

Recovery of potential adjunct LAB from Egyptian ripened cheese

The ability of adjunct cultures to enhance the development of typical flavor in cheese gives rise to the possibility of their association with ripened cheese with favorable flavor. Samples of Egyptian ripened cheese were thus collected and assessed for their flavor. Only samples with "good" or "acceptable" flavor were further analyzed as a potential source of adjunct LAB.

Table 1 shows the results of flavor assessment of pickled Domiatti cheese and Ras cheese samples. Out of 54 cheese samples, 9 and 6 samples of pickled Domiatti cheese and Ras cheese, respectively were found by the panelists to have "good" flavor. Whereas, 8 and 7 samples of pickled Domiatti cheese and Ras cheese, respectively showed "acceptable" flavor. The rest of cheese samples were found to have "poor" and "unacceptable" flavor. Only cheese samples with"good" and "acceptable" flavor were further used for the isolation of potential adjunct LAB.

Table 1. Flavor assessment of pickled Domiatti cheese and Ras cheese samples.

Sensory evaluation scale	Number of cheese samples		
	Pickled Domiatti cheese	Ras cheese	Total
Good (76-100)	9	6	15
Acceptable (51-75)	8	7	15
Poor (26-50)	13	4	17
Unacceptable (0-25)	3	4	7
Total	33	21	54

Table 2 shows the number of suspected and potential LAB cultures isolated from pickled Domiatti cheese and Ras cheese samples with "good" and "acceptable" flavor. A total of 162 suspected LAB isolates could be recovered from those samples and were subjected to further confirmatory testing that identified 37 and 4 potential LAB cocci and lactobacilli, respectively. Those potential LAB cultures were Gram-positive, catalase-negative, and could coagulate milk. They were further confirmed to belong to the *Enterococcus* (37 isolates) and *Lactobacillus* (4 isolates) genera using the physiological examinations shown in Table 3. It could be noted that *Enterococcus* isolates were variable in their ability to grow at 45 °C, and in the presence of 6.5% NaCl, which has been previously reported by Devriese and Pot (1995). For instance, *Enterococcus dispar* and *Enterococcus sulfureus* were found not to be able to grow at 45 °C. Also *Enterococcus cecorum*, *Enterococcus columbae*, *Enterococcus avium*, and related species are often negative in their ability to grow in 6.5% NaCl.

Table 2. Isolation and preliminary identification of lactic acid bacteria from cheese samples.

Samples	No. of samples	No. of suspected LAB isolates	Potential LAB isolates	
			Cocci	Lactobacilli
Pickled Domiatti cheese	17	60	5	4
Ras cheese	13	102	32	0
Total	30	162	37	4

Physiological variability was also observed in the *Lactobacillus* isolates in terms of their ability to grow at 45 °C and in the presence of 6.5% NaCl (Table 3), which was consistent with previous reports (Von Wright and Axelsson, 1998; Vasiee *et al.*, 2014). This variability could be due to the large number of approximately 80 diverse species included within *Lactobacillus* that made it the largest LAB genus ((Euzèby,1997; Von Wright and Axelsson, 1998).

The inability of the *Enterococcus* and *Lactobacillus* isolates to produce CO_2 from glucose as shown in Table 3 indicates that they have a homo-fermentative pathway of carbohydrate. This presents them as suitable candidates for use as adjunct cultures given that gas production by hetero-fermentative cultures leads to quality defects in cheese (El-Soda *et al.*, 2000).

Table 3. Confirmatory physiological examinations of potential LAB isolates from cheese samples.

Potential LAB Isolates (No. of Isolates)	Growth at 10°C	Growth at 45°C	Growth at pH 9.6	Growth in 4%NaCl	Growth in 6.5% NaCl	Growth on BEA medium	CO_2 production from glucose	Results of Identification (No. of Isolates)
Cocci (37)	+	±	+	+	±	+	-	*Enterococcus* spp. (37)
Lactobacilli (4)	+	±	+	+	±	-	-	*Lactobacillus* spp.(4)

Enterococcus and *Lactobacillus* isolates were further identified to the species level employing diverse physiological and biochemical examinations as shown in Tables 4 and 5. Results were interpreted according to Devriese and pot (1995) and Manero and Blanch (1999) for *Enterococcus* and Badis *et al*. (2004) and Vasiee *et al*., (2014) for *Lactobacillus*. Based on this, *Enterococcus* isolates were found to involve diverse species of *Ent. faecalis* (2 isolates), *Ent. faecium* (7 isolates), *Ent. gallinarum* (2 isolates), *Ent. durans* (1 isolate), *Ent. mundtii* (2 isolates), *Ent. casseiliflavus* (2 isolates), *Ent. pseudoaavium* (12 isolates), and *Enterococcus* spp. (9 isolates). Whereas, *Lactobacillus* isolates could be identified as *Lb. plantarum* (1 isolate) and *Lactobacillus* spp. (3 isolates).

These results indicated that diverse species of *Enterococcus* and *Lactobacillus* could exist in Domiatti and Ras cheese samples, which was also reported in previous studies. For instance, El-Soda *et al*. (2003) and Ayad *et al*. (2004 & 2006) detected different species of *Lactococcus*, *Lactobacillus*, *Enterococcus*, *Streptococcus* and *Pediococcus* in Domiatti cheese, Ras cheese, Mish, Zabady and Laban Rayeb. The present results also show that *Enterococcus* isolates were the most frequently isolated

LAB from Domiatti and Ras cheese samples. This agreed with previous studies presenting *Enterococcus* as a frequent non-starter lactic acid bacteria species in various cheese types (Süßmuth, 1995; Giraffa, 2003; Franciosi *et al*., 2009; El-Soda *et al*., 2011). For example, Süßmuth (1995) found that the majority of 300 LAB strains isolated from Domiatti and Ras cheeses were identified as *Enterococcus* with only 3.5% of them belonging to *Lactococcus* and 1.5% belonging to *Streptococcus thermophilus* and *Streptococcus bovis*. A potential explanation for this is the ability of *Enterococcus* to survive under stress conditions of heat, salt, and acidity during cheese making (Flahaut *et al*., 1996; Jokovic *et al*., 2008).

Table 4. Physiological identification of *Enterococcus* isolates recovered from cheese samples.

No. of isolates	Growth at 45°C	Growth in 6.5%NaCl	Carbohydrate Fermentation									Pyruvate utilization	Motility	H_2S production	Yellow pigment production	Results of Identification
			Lac	Glu	Suc	Sor	Man	Fru	Mal	Tre	Xyl					
2	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	*Ent. faecalis*
7	+	+	+	+	+	-	+	+	+	+	-	-	-	-	-	*Ent. faecium*
2	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	*Ent. gallinarum*
1	+	+	+	+	+	-	-	+	+	+	-	-	-	-	-	*Ent. durans*
2	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	*Ent. mundtii*
2	+	+	+	+	+	+	+	+	+	+	-	-	+	-	+	*Ent. casseiliflavus*
12	+	-	+	+	+	+	+	+	+	+	-	±	-	-	-	*Ent. pseudoavium*
9	-	±	+	+	+	+	+	+	+	+	-	±	-	-	-	*Enterococcus* spp.

Lac: Lactose, Glu: Glucose, Suc: Sucrose, Sor: Sorbitol, Man: Mannitol, Fru: Fructose, Mal: Maltose, Tre: Trehalose, Xyl: L-Xylose.

Table 5. Physiological identification of *Lactobacillus* isolates recovered from cheese samples.

No. of isolates	Growth at pH 9.6	Growth at 45°C	Growth in 6.5% NaCl	Carbohydrate fermentation								Results of identification
				Lac	Glu	Suc	Sor	Man	Fru	Mal	Xyl	
1	+	-	+	+	+	+	+	+	+	+	+	*Lb. plantarum*
2	+	+	+	+	+	+	-	-	-	-	+	*Lactobacillus* spp.
1	+	+	-	+	+	+	+	-	+	-	+	*Lactobacillus* spp.

Lac: Lactose, Glu: Glucose, Suc: Sucrose, Sor: Sorbitol, Man: Mannitol, Fru: Fructose, Mal: Maltose, Xyl: L-Xylose.

Technological properties of potential LAB adjuncts

Acidity development

Acid production is an important criterion for LAB cultures to be selected as a starter or adjunct culture (Sarantinopoulos *et al.*, 2001). Strains with strong acidifying activities are mainly employed as primary starters in dairy fermentations. Whereas, LAB cultures with moderate or poor acidifying activities are preferred as adjunct cultures to avoid excess acidity production. Therefore, all identified *Enterococcus* and *Lactobacillus* isolates in this study were tested for their ability to produce acid in milk after 24 h of incubation at 37 ºC in sterilized reconstituted skim milk (RSM).

As shown in Table 6, the majority of *Enterococcus* isolates had an acidifying activity higher than 0.5% (titratable acidity %). However, the isolates showed variability in acid production that ranged from a titratable acidity of 0.43% and pH 5.35 by the *Enterococcus* spp. isolate number 34 to a titratable acidity of 0.9% and pH 4.61 by the *Enterococcus pseudoavium* isolate number 25.

Table 7 shows the acidity development by *Lactobacillus* cultures isolated from cheese. It could be seen that the acid production of *Lactobacillus* isolates ranged from a titratable acidity of 0.45% and pH 5.53 by the *Lactobacillus* spp. isolate number 4 to a titratable acidity of 0.51% and pH 5.34 by the *Lactobacillus* spp. isolate number 2. These results showed that *Lactobacillus* isolates did not have strong ability for milk acidification. This was consistent with previous studies reporting low acidifying activity of *Lactobacillus plantarum*, and other *Lactobacillus* spp. isolates grown in RSM at 37 ºC (Morea *et al*., 1998; Herreros *et al*., 2003; Nieto-Arribas *et al*., 2009).

Proteolytic activity

Suitable proteolytic activity is a significant property for adjunct cultures that allows them to break down milk proteins and produce short peptides and free amino acids leading to flavor and texture development in cheese (Yvon, 2006). The proteolytic activity of *Enterococcus* and *Lactobacillus* isolates recovered from cheese samples in the present study was thus examined by using two methods: a) platting on a nutritious medium (TSA or M17 agar supplemented with milk), b) a colorimetric method to assess the concentration of protein degradation products determined as tyrosine.

Table 6 shows the results of the proteolytic activity of the 37 *Enterococcus* cultures isolated from cheese samples by using both methods. Out of the 37 *Enterococcus* isolates, only 8 isolates showed positive proteolytic activities by platting on TSA supplemented with milk. These isolates belonged to *Ent. faecalis* (isolate number 2), *Ent. faecium* (isolate number 4), *Ent. mundtii* (isolate number 14), *Ent. casseiliflavus* (isolate number 16), *Ent. pseudoavium* (isolates number 19, 21, 25), and *Enterococcus* ssp. (isolate number 37).

The assessment of the proteolytic activity of the 37 *Enterococcus* isolates by using the colorimetric method showed variable results among isolates (Table 6). Based on this variability, *Enterococcus* isolates could be categorized into three groups. The first group involved isolates of a strong proteolytic activity of 100-

200 µg tyrosine/ml. This group included 3 isolates belonging to *Ent. faecalis* (isolate number 2), *Ent. faecium* (isolate number 4), and *Ent. mundtii* (isolate number14). The second group contained isolates that had moderate proteolytic activity of 50-100 µg tyrosine/ml. This group included 4 isolates of *Ent. pseudoavium* (isolates number 19, 21 and 25), and *Enterococcus* ssp. (isolate number 37). The third group involved the rest of the *Enterococcus* isolates that showed a low proteolytic activity of less than 50 µg tyrosine/ml.

The proteolytic activities of the *Lactobacillus* isolates recovered from cheese samples are presented in Table 7. It could be noted that the 4 *Lactobacillus* isolates exhibited proteolytic activity as indicated by platting on M17 agar supplemented with milk. They also showed generally low proteolytic activity values ranging from 32.1 to 45.7 µg tyrosine/ml by using the colorimetric method.

These results indicated that *Enterococcus* and *Lactobacillus* cultures could vary greatly in their proteolytic activity, which highlights the importance of examining them for this trait before use as starter cultures or adjuncts. While strong proteolytic strains of LAB could be preferred for accelerating ripening, they may also lead to the production of bitter peptides in cheese due to excessive proteolysis (Buffa *et al*., 2005).

Table 6. Technological properties of *Enterococcus* cultures isolated from cheese samples.

Iso. N	Microorganisms	Acidifying activity		Proteolytic activity		Lipolytic activity	
		T. A %*	pH	Platting on TSA supplemented with milk	Colorimetric method (µg Tyrosine/mL)	Platting on TSA supplemented with cream fat	TVFFA**
1	*Ent. faecalis*	0.6	4.93	-	0.53	-	3.4
2	*Ent. faecalis*	0.8	4.77	+	178.42	+	4.1
3	*Ent. faecium*	0.5	5.32	-	0.53	+	4
4	*Ent. faecium*	0.88	4.69	+	117.84	-	3.3
5	*Ent. faecium*	0.7	4.9	-	0.82	-	3.4
6	*Ent. faecium*	0.7	4.87	-	0.44	-	3.1
7	*Ent. faecium*	0.74	4.77	-	3.42	-	3.2
8	*Ent. faecium*	0.72	4.94	-	4.48	+	4
9	*Ent. faecium*	0.8	4.76	-	0.92	-	3.4
10	*Ent. gallinarum*	0.69	4.96	-	2.46	-	3.4
11	*Ent. gallinarum*	0.68	4.95	-	0.63	+	4.4
12	*Ent. durans*	0.69	4.73	-	7.94	-	3.2
13	*Ent. mundtii*	0.45	5.36	-	0.44	-	3.2
14	*Ent. mundtii*	0.84	4.73	+	103.61	+	4.3
15	*Ent. casseiliflavus*	0.63	4.87	-	9	-	3.5
16	*Ent. casseiliflavus*	0.65	4.81	+	31.88	+	4.2
17	*Ent. pseudoavium*	0.68	4.93	-	0.34	-	3.2
18	*Ent. pseudoavium*	0.54	5.18	-	5.92	+	4
19	*Ent. pseudoavium*	0.72	4.77	+	98.23	+	3.8
20	*Ent. pseudoavium*	0.5	5.31	-	10.73	-	3.5
21	*Ent. pseudoavium*	0.65	4.85	+	77.46	-	3.2
22	*Ent. pseudoavium*	0.48	5.4	-	1.4	+	3.9
23	*Ent. pseudoavium*	0.5	5.36	-	1.1	-	3.4
24	*Ent. pseudoavium*	0.55	5.1	-	2.65	-	3.3
25	*Ent. pseudoavium*	0.9	4.61	+	78.71	+	5.2
26	*Ent. pseudoavium*	0.74	4.76	-	1.4	-	3.1
27	*Ent. pseudoavium*	0.7	4.78	-	0.82	-	3.2
28	*Ent. pseudoavium*	0.7	4.86	-	0.53	-	3.1
29	*Enterococcus* spp.	0.5	5.29	-	0.44	-	3.5
30	*Enterococcus* spp.	0.59	5.08	-	0.44	-	3.4
31	*Enterococcus* spp.	0.52	5.08	-	0.53	+	3.8
32	*Enterococcus* spp.	0.74	4.86	-	3.13	+	3.9
33	*Enterococcus* spp.	0.65	4.8	-	5.92	+	4.2
34	*Enterococcus* spp.	0.43	5.35	-	0.92	-	3.1
35	*Enterococcus* spp.	0.59	5.18	-	1.01	+	3.9
36	*Enterococcus* spp.	0.6	5.16	-	0.44	+	4.3
37	*Enterococcus* spp.	0.7	4.98	+	91.11	+	4.2

T.A%* : Titratable Acidity percent.
TVFFA : Total volatile free fatty acids (results were expressed as the volume of NaOH 0.1 N used in the titration of TVFFA in 100g sample.**

Lipolytic activity

Enterococcus and *Lactobacillus* isolates recovered from cheese samples in the current study were further screened for their lipolytic activity by platting on a nutritious medium (TSA or M17 agar) supplemented with 0.4% cream fat. They were also examined for their ability to produce volatile free fatty acids from milk by using the distillation method described by Kosikowski (1978).

Table 6 shows the results of lipolytic activity of 37 *Enterococcus* isolates by platting on TSA supplemented with 0.4% cream fat. Using this method,

lipolytic activity could be evidenced by 16 *Enterococcus* isolates. These isolates included *Ent. faecalis* isolate number 2, *Ent. faecium* isolates number 3 and 8, *Ent. gallinarum* isolate number 11, *Ent. mundtii* isolate number 14, *Ent. casseiliflavus* isolate number 16, *Ent. pseudoavium* isolates number 18, 19, 22 and 25, and *Enterococcus* spp. isolates number 31, 32, 33, 35, 36 and 37. Those isolates showed total volatile fatty free acids (TVFFA) determinations of 3.8-5.2. *Lactobacillus* isolates also showed lipolytic activity on M17 agar supplemented with 0.4% cream fat and were able to produce TVFFA of 3.7-3.9 (Table 7).

Taken together, these results showed that both *Enterococcus* and *Lactobacillus* isolates recovered from traditional Egyptian ripened cheese could variably express lipolytic activity. This lipolytic activity allows the breakdown of milk fat that releases fatty acids to enhance cheese flavor, which supports the use of those cultures as adjuncts in cheese making. However, strong lipolytic isolates have to be avoided since they may lead to the development of rancid flavor.

Table 7. Technological properties of *Lactobacillus* cultures isolated from cheese samples.

Iso. N	Microorganism	Acidifying activity		Proteolytic activity		Lipolytic activity	
		T.A%*	pH	Platting on TSA supplemented with milk	Colorimetric method (µg tyrosine/mL)	Platting on TSA supplemented with cream fat	TVFFA**
1	*Lb. plantarum*	0.48	5.42	+	33.2	+	3.8
2	*Lactobacillus* spp.	0.51	5.34	+	37.1	+	3.9
3	*Lactobacillus* spp.	0.48	5.47	+	45.7	+	3.8
4	*Lactobacillus* spp.	0.45	5.53	+	32.1	+	3.7

T.A%* : Titratable Acidity percent.
TVFFA : Total volatile free fatty acids (results were expressed as the volume of NaOH 0.1N used in the titration of TVFFA in 100g sample.**

Safety assessment of potential LAB adjuncts

Antibiotic resistance

The antibiotic resistance of *Enterococcus* and *Lactobacillus* cultures isolated from cheese samples was determined against 9 antibiotics using the Kirby-Bauer disc-diffusion method (Fortina *et al*., 2008). Table 8 shows the results of antibiotic resistance of *Enterococcus* cultures isolated from cheese samples. All *Enterococcus* isolates were found to be resistant to oxacillin (1 µg) (Table 8). This was followed by clindamycin (2 µg), lincomycin (10 µg), penicillin (10 µg), streptomycin (10 µg), kanamycin (10 µg), ampicillin (10 µg), chloramphenicol (30 µg) and gentamycin (10 µg) that were resisted by 78%, 73%, 65%, 51%, 19%, 16%, 11% and 5% of the isolates, respectively (Table 8). These results could be expected given that foodborne *Enterococcus* cultures were previously shown to possess a broad spectrum of antibiotic resistance (Klare *et al*., 2003). Those antibiotic-resistant cultures were detected in meat products, dairy products, and ready-to-eat foods (Franz *et al*., 1999; Giraffa, 2002).

All *Lactobacillus* cultures isolated from cheese samples in this study were also resistant to oxacillin (1 µg) (Table 9). One *Lactobacillus* spp. isolate was also resistant to streptomycin (10 µg). However, the isolates were sensitive to the other seven antibiotics. There have been a few studies reporting the association of antibiotic-resistant lactobacilli with dairy products. For instance, Katla *et al*. (2001) showed that only one out of 189 *Lactobacillus* isolates recovered from Norwegian dairy products was resistant to streptomycin.

The antibiotic-resistance of *Enterococcus* and *Lactobacillus* isolates cultured from ripened Egyptian cheese in this study has to be considered as an important criterion for the selection of those isolates for use as adjuncts in cheesemaking. Incorporating antibiotic-resistant adjuncts in cheese may lead to horizontal transfer of genetic elements responsible for antibiotic resistance from those cultures to pathogenic strains in the human gut (El-Sharoud *et al*. 2015). This presents serious health risk complicating medical treatment of bacterial infections.

Biogenic amine production

Biogenic amines are organic compounds produced by the decarboxylation of amino acids by microorganisms. They have been associated with disease symptoms including headaches, palpitations and vomiting (Brink *et al.*, 1990; Halász *et al.*, 1994). Histamine and tyramine, which are produced by enzymatic decarboxylation of histidine and tyrosine, respectively have been the most frequently studied biogenic amines due to their toxicological effects (EFSA, 2011; Linares *et al.*, 2012). The present study thus examined the production of those two biogenic amines by *Enterococcus* and *Lactobacillus* cultures isolated from Egyptian ripened cheese samples. It could be seen in Table 10 that a total of 6 isolates of *Enterococcus* including *Ent. faecium* (2 isolates), *Ent. pseudoavium* (3 isolates) and *Enterococcus* spp. (1 isolate) could produce histamine. Whereas, a higher number of 13 isolates involving *Ent. faecium* (1 isolate), *Ent. durans* (1 isolate), *Ent. mundtii* (1 isolate), *Ent. casseiliflavus* (2 isolates), *Ent. pseudoavium* (4 isolates) and *Enterococcus* spp. (4 isolates) were able to produce tyramine (Table 10).

Table 8. Antibiotic resistance of *Enterococcus* cultures isolated from cheese samples.

Iso.N	Microorganism	Antibiotics								
		AMP	**P**	**DA**	**CN**	**K**	**S**	**MY**	**C**	**OX**
1	*Ent. faecalis*	R	R	R	S	S	S	R	S	R
2	*Ent. faecalis*	R	R	R	S	S	S	R	S	R
3	*Ent. faecium*	S	S	S	S	R	R	S	R	R
4	*Ent. faecium*	S	R	R	S	S	R	R	S	R
5	*Ent. faecium*	R	R	R	S	S	S	R	S	R
6	*Ent. faecium*	S	S	R	R	R	I	S	R	R
7	*Ent. faecium*	S	R	R	S	S	S	R	S	R
8	*Ent. faecium*	S	S	S	S	R	R	S	S	R
9	*Ent. faecium*	S	S	R	S	R	R	S	S	R
10	*Ent. gallinarum*	S	S	S	S	S	R	S	S	R
11	*Ent. gallinarum*	S	R	R	S	S	S	R	S	R
12	*Ent. durans*	S	R	R	S	S	S	R	S	R
13	*Ent. mundtii*	S	R	R	S	S	R	R	S	R
14	*Ent. mundtii*	S	R	R	S	S	S	R	S	R
15	*Ent. casseiliflavus*	S	R	R	S	S	R	R	S	R
16	*Ent. casseiliflavus*	R	I	R	S	S	I	R	S	R
17	*Ent. pseudoavium*	S	R	R	S	R	R	R	R	R
18	*Ent. pseudoavium*	S	S	S	I	R	R	S	I	R
19	*Ent. pseudoavium*	R	R	R	S	S	S	R	S	R
20	*Ent. pseudoavium*	S	R	R	S	S	S	R	S	R
21	*Ent. pseudoavium*	S	R	R	S	S	S	R	S	R
22	*Ent. pseudoavium*	S	S	S	S	S	R	S	S	R
23	*Ent. pseudoavium*	S	S	S	S	S	R	S	S	R
24	*Ent. pseudoavium*	S	R	R	S	S	S	R	S	R
25	*Ent. pseudoavium*	S	R	R	S	S	R	R	S	R
26	*Ent. pseudoavium*	S	R	R	S	S	R	R	S	R
27	*Ent. pseudoavium*	S	S	R	R	S	R	R	S	R
28	*Ent. pseudoavium*	S	R	R	S	S	R	R	S	R
29	*Enterococcus* spp.	R	R	R	S	S	S	R	S	R
30	*Enterococcus* spp.	S	S	R	S	S	R	R	S	R
31	*Enterococcus* spp.	S	R	R	S	S	S	R	S	R
32	*Enterococcus* spp.	S	R	R	S	S	S	R	S	R
33	*Enterococcus* spp.	S	R	R	S	S	S	R	S	R
34	*Enterococcus* spp.	S	S	S	S	R	R	S	R	R
35	*Enterococcus* spp.	S	R	R	S	S	S	R	S	R
36	*Enterococcus* spp.	S	S	S	S	S	R	S	S	R
37	*Enterococcus* spp.	S	R	R	S	S	R	R	S	R
	Number of resistant isolate	6	24	29	2	7	19	27	4	37
	(%)	(16%)	(65%)	(78%)	(5%)	(19%)	(51%)	(73%)	(11%)	(100%)

***AMP: ampicillin (10 µg), P: penicillin(10 µg), DA: clindamycin (2 µg), CN: gentamycin (10 µg), K: kanamycin (10 µg), S: streptomycin (10 µg), MY: lincomycin (10 µg), C: chloramphenicol (30 µg), and OX: oxacillin (1 µg).**

Table 9. Antibiotic resistance of *Lactobacillus* cultures isolated from cheese samples.

Isolate number	Microorganism	Antibiotics*								
		AMP	**P**	**DA**	**CN**	**K**	**S**	**MY**	**C**	**OX**
1	*Lactobacillus plantarum*	S	S	S	S	S	S	S	S	R
2	*Lactobacillus* spp.	S	S	S	S	S	S	S	S	R
3	*Lactobacillus* spp.	S	S	S	S	S	S	S	S	R
4	*Lactobacillus* spp.	S	S	S	S	S	R	S	S	R
	Number of resistant isolate	0	0	0	0	0	1	0	0	4
	(%)	(0%)	(0%)	(0%)	(0%)	(0%)	(25%)	(0%)	(0%)	(100%)

***AMP: ampicillin (10 µg), P: penicillin(10 µg), DA: clindamycin (2 µg), CN: gentamycin (10 µg), K: kanamycin (10 µg), S: streptomycin (10 µg), MY: lincomycin (10 µg), C: chloramphenicol (30 µg), and OX: oxacillin (1 µg).**

On contrast, none of the *Lactobacillus* isolates were able to produce histamine or tyramine (*data not shown*). These results agree with previous studies reporting histamine and tyramine among the most important biogenic amines in dairy foods (EFSA, 2011; Linares *et al.*, 2012). The results also present biogenic amine production as another safety trait to be considered when selecting LAB for use as adjuncts given the adverse health impact of those compounds.

Table 10. Biogenic amine production and hemolytic activity of *Enterococcus* isolates recovered from cheese samples.

Isolate number	Microorganisms	Biogenic amines production		Hemolytic activity
		Histamine	Tyramine	
1	*Ent. faecalis*	-	-	+
2	*Ent. faecalis*	-	-	-
3	*Ent. faecium*	-	-	-
4	*Ent. faecium*	-	+	-
5	*Ent. faecium*	-	-	-
6	*Ent. faecium*	-	-	+
7	*Ent. faecium*	+	-	-
8	*Ent. faecium*	+	-	+
9	*Ent. faecium*	-	-	+
10	*Ent. gallinarum*	-	-	+
11	*Ent. gallinarum*	-	-	+
12	*Ent. durans*	-	+	-
13	*Ent. mundtii*	-	+	+
14	*Ent. mundtii*	-	-	+
15	*Ent. casseiliflavus*	-	+	+
16	*Ent. casseiliflavus*	-	+	+
17	*Ent. pseudoavium*	+	+	-
18	*Ent. pseudoavium*	+	-	-
19	*Ent. pseudoavium*	-	-	-
20	*Ent. pseudoavium*	-	-	-
21	*Ent. pseudoavium*	-	-	-
22	*Ent. pseudoavium*	-	+	-
23	*Ent. pseudoavium*	-	+	-
24	*Ent. pseudoavium*	-	-	-
25	*Ent. pseudoavium*	-	+	-
26	*Ent. pseudoavium*	+	-	-
27	*Ent. pseudoavium*	-	-	-
28	*Ent. pseudoavium*	-	-	-
29	*Enterococcus* spp.	-	-	-
30	*Enterococcus* spp.	-	+	+
31	*Enterococcus* spp.	-	-	-
32	*Enterococcus* spp.	-	-	-
33	*Enterococcus* spp.	-	-	+
34	*Enterococcus* spp.	+	+	+
35	*Enterococcus* spp.	-	+	+
36	*Enterococcus* spp.	-	-	-
37	*Enterococcus* spp.	-	+	-

Hemolytic activity

The ability of bacterial cells to lyse blood cells (hemolytic activity) is a highly virulence trait that is frequently associated with pathogenic bacteria (Tyne *et al*., 2013). Adjunct cultures intended for use in cheese making must therefore be unable to express hemolytic activity. The *Enterococcus* and *Lactobacillus* isolates recovered from Egyptian ripened cheese samples is this study was examined for hemolytic activity. A total of 14 isolates of *Enterococcus* cultures were found to be hemolytic (Table 10). Those isolates included *Ent. faecalis* (1 isolate), *Ent. faecium* (3 isolates), *Ent. gallinarum* (2 isolates), *Ent. mundtii* (2 isolates), *Ent. casseiliflavus* (2 isolates) and *Enterococcus* spp. (4 isolates). Whereas, none of the *Lactobacillus* isolates were able to express hemolytic activity (*data not shown*).

These results are consistent with previous studies reporting hemolytic activity by different *Enterococcus* strains (Antalek *et al*., 1995; De Vuyst *et al.*, 2003; Semedo *et al.*, 2003). On contrast, hemolytic activity could not be detected in *Lactobacillus* strains isolated from different sources (Anas *et al.*, 2008; Pisano *et al.*, 2014; Leite *et al.*, 2015).

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عزل و تقدير الخواص التكنولوجية و صفات الأمان للمزارع المساعدة من بكتريا حامض اللاكتيك

الطاهرة محمد أحمد عمار ، عبد الوهاب الشاذلي خليل ، سمر أحمد زلمة و وليد محمود الشارود
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المزارع المساعدة هي ميكروبات لا تدخل في تكوين البادئات الأساسية في صناعة الجبن ولكنها تساعد علي ظهور النكهة والقوام المفضلين للجبن أثناء التسوية. ولقد تم تصميم هذه الدراسة لعزل وتعريف سلالات من بكتريا حامض اللاكتيك من الجبن الدمياطي الخزين والجبن الرأس وذلك لإستخدامها كمزارع مساعدة، حيث تم تجميع ٥٤ عينة جبن تضمنت ٣٣ عينة جبن دمياطي خزين و٢١ عينة جبن رأس من مدينة المنصورة والقري المحيطة بها وتم تقييم نكهة الجبن في هذه العينات بواسطة محكمين مناسبين، ونظراً لارتباط المزارع المساعدة بالنكهة المعتادة للجبن فإن العينات التي تم تقييم نكهتها بتقديرات "جيد" و"مقبول" تم استخدامها كمصدر لعزل المزارع المساعدة. وقد تم عزل ١٦٢ مزرعة مشكوك فيها كبكتريا حامض لاكتيك من هذه العينات، وتم تعريف ٣٧ عزلة منها علي أنها تنتمي لجنس الانتيروكوكس *Enterococcus* و ٤ عزلات علي أنها تنتمي لجنس اللاكتوبسلس *Lactobacillus* و باجراء الأختبارات الفسيولوجية المختلفة لتحديد النوع البكتيرى وجد أن ٣٧ عزلة التابعة لجنس الأنتيروكوكس تنتمى للأنواع الآتية: انتيروكوكس فيكالس (عزلتين) و انتيروكوكس فيشيم (٧ عزلات) و انتيروكوكس جالينرم (عزلتين) و انتيروكوكس ديورنس (عزلة) و انتيروكوكس ميندتاى (عزلتين) و انتيروكوكس كاسيفلافيس (عزلتين) و انتيروكوكس سيدواوفيم (١٢ عزلة) بالإضافة إلي ٩ عزلات تنتمى لنوع غير معروف من الأنتيروكوكس. كما تبين ان عزلات جنس اللاكتوبسلس تنتمى للاكتوبسلس بلانترم (عزلة واحدة) و ٣ عزلات تنتمى لنوع غير معروف من اللاكتوبسلس. وقد تم دراسة الخواص التكنولوجية لهذه العزلات و التى تشمل انتاج الحموضة و قدرتها على تحليل البروتين و الدهن. وقد أظهرت العزلات تنوعاً في هذه الخواص، كما تم دراسة خواص الأمان لهذه العزلات و التى تشمل مقاومتها للمضادات الحيوية و انتاجها للأمينات الحيوية و كذلك قدرتها على تحليل الدم. و قد اظهرت عزلات الانتيروكوكس مقاومةً لعدة مضادات حيوية كما استطاعت انتاج الأمينات الحيوية هستامين وتيرامين، كما تمكن بعضها من تحليل الدم، بينما أظهرت عزلات اللاكتوباسلس مقاومة لنوعين فقط من المضادات الحيوية ولم تستطع انتاج الهستامين أو التيرامين، كما أنها لم تتمكن من تحليل الدم. وبوضع هذه النتائج معاً فإن هذه الدراسة تقدم مجموعة من عزلات الانتيروكوكس واللاكتوباسلس التي يمكن تقييمها للاستخدام كمزارع مساعدة بناءً علي صفاتها التكنولوجية والخواص المرتبطة بالأمان الصحي.