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TECHNOLOGICAL CHARACTERISTICS AND ANTIBACTERIAL ACTIVITIES OF *Lactococcus lactis* subsp. *Lactis* ISOLATES RECOVERED FROM LABAN RAYEB AND KARIESH CHEESE

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ABSTRACT

Lactococcus lactis subsp. *lactis* is widely used as a starter in the preparation of fermented dairy products. Extensive amounts of commercial starters containing this organism are annually imported from Western countries, which represents a burden on the national dairy industries and retards its development. This study aimed to isolate the organism from Laban Rayeb and Kariesh cheese, which are locally available traditional fermented dairy products that contain diverse bacterial floras. The study also considered characterizing the technological characteristics and antibacterial activities of the resultant isolates. Thirty *Lactococcus* spp isolates could be recovered from 68 samples of Laban Rayeb and Kariesh cheese and identified as *Lac. lactis* subsp. *lactis*. Three isolates of the same organism were also recovered from commercial starters and compared with those cultured from Laban Rayeb and Kariesh cheese. The total of the 33 isolates were assessed for their technological characteristics and antibacterial activities. Results showed that there were isolates of *Lac. lactis* subsp. *lactis* recovered from Laban Rayeb and Kariesh cheese that produced titratable acidity and expressed proteolytic activities significantly higher ($P<0.05$) than isolates obtained from commercial starters. Some of the isolates were also able to produce exopolysaccharides, but produced less acidity. The isolates were further found to show antibacterial activities against *Staphylococcus aureus* and *Salmonella* ser. Typhimurium. Together, these results show that it is possible to select isolates of *Lac. lactis* subsp. *lactis* recovered from Laban Rayeb and Kariesh cheese and use them in the preparation of dairy products instead of imported starter cultures.

INTRODUCTION

Starter cultures are of great significance to dairy industries given their vital roles in the manufacture and flavor and texture development of fermented dairy foods. Global sales of dairy starters amounted approximately 4.8 million US Dollars in 2005, with most dairy starter production facilities being based in Europe and USA (Cogan *et al*., 2007). While dairy industries represent an important food production sector in Egypt, there is a lack of dairy starter factories in this country. Massive amounts of dairy starters are annually purchased and imported from countries like Denmark and France representing a burden on the development and independence of dairy industries in Egypt. A prime reason for the lack of national dairy starter factories is the lack of collections of isolated and well-characterized industrial bacterial strains that could be used as dairy starters.

Lactic acid bacteria (LAB) are the most frequently used microorganisms in dairy starters. LAB involve several species, of which the genus *Lactococcus* is heavily employed in the preparation of fermented dairy products (Batt, 1999). The genus *Lactococcus* is relatively new and involves microorganisms that previously belonged to the genera *Streptococcus* and *Lactobacillus*. It involves 5 species; *Lac. garvieae, Lac. lactis, Lac. piscium, Lac. plantarum* and *Lac. raffinolactis*. Within the species *Lac. lactis*, the subspecies *L. lactis* subsp. *lactis* and *cremoris* are the most widely used in dairy fermentations. Lactococci are employed either as single strain starters or as part of a multiple-strain starter mixture. The latter may consist of different strains from a single species, or multiple strains of different species.

Laban Rayeb and Kariesh cheese are two related traditional Egyptian dairy products prepared in the rural areas. The preparation of these products is frequently carried out under artisanal conditions and involves the use of raw milk. This allows the presence of diverse bacterial floras in these products, which provides an opportunity for isolating diverse LAB strains, some of which may be of improved industrial performance. The present study was therefore designed to isolate and characterize strains of *Lac. lastis* subsp. *lactis* associated with Laban Rayeb and Kariesh cheese. Strain characterization was based on technological traits and antibacterial activities of isolated lactococci in comparison with *Lac. lactis* isolates cultured from imported commercial dairy starters. This aimed to initiate the establishment of a local collection of well-characterized and selected strains of lactococci that could be employed in dairy industries.

MATERIALS AND METHODS

Sample collection

Sixty-eight (68) samples of Laban Rayeb (n= 28) and Kariesh cheese (n= 40) were randomly and aseptically collected from rural areas in the Dakahlia and Domiati governorates. Two commercial mesophilic starters produced by Danisco (France) and Christian Hansen (Denmark) were also purchased and used for the isolation of *Lac. lactis* subsp. *lactis*.

Isolation of lactococci

The isolation of lactococci from the examined products depended on the method described by Mannu & Paba (2002) as follows. Serial dilutions (1:9) of Laban Rayeb and Kariesh cheese samples (10 g) were prepared in sterile peptone/saline solution (0.1% peptone, 0.85% NaCl) and plated onto M17 agar plates (Oxoid, Basingstoke, UK) and incubated at 30°C for 3 d under anaerobic conditions (Gas Generating Kit, Oxoid). After the incubation period, a representative number of colonies were randomly selected from M17 plates and examined by Gram staining and catalase reaction. Gram-positive, catalase-negative cocci isolates were further examined for their growth at various temperatures (10°C, 45°C, and 60°C), tolerance of different salt levels (2%, 4% and 6.5% NaCl), production of gas from glucose and hydrolysis of arginine (Harrigan & McCance, 1990).

Speciation of lactococci

The carbohydrate fermentation profiles of the selected isolates were investigated using API 50 CHL miniaturized kits according to the manufacturer's instructions (API System, Bio-Merieux, France). Species were determined tentatively through the use of APILAB PLUS (Version 3.2.2., Bio-Merieux) and standard taxonomic descriptions from Wood & Holzapfel (1995).

Assessment of acid production

Acid production of identified *Lac. lactis* isolates were assessed in sterilized reconstituted skim milk (RSM) (10% total solids) by inoculating milk with 2% (v/v) of an overnight culture of each isolate grown in M17 broth. Inoculated RSM was incubated at 30°C for 24 h and titratable acidity was measured.

Examination of exopolysaccharide (EPS) production

EPS production was evaluated in ruthenium red milk (RRM) plates consisting of 0.5% yeast extract, 10% skim milk powder, 1% sucrose, 1.5% agar, and 0.08 g ruthenium red per liter (Mora *et al*., 2002). EPS-producing strains developed white colonies, while EPS-nonproducing strains appeared as red colonies (Stingele *et al*., 1996).

Assessment of proteolytic activity

The proteolytic activity of *Lac. lactis* isolates grown in RSM (10% TS) was determined according to the methods of Citti *et al*. (1963) and Moreira *et al*. (2003). Proteins were precipitated with TCA (117.6 g/Lt). The TCA soluble N was determined through the use of Folin's reagent. The absorbance of the characteristic blue color was measured at 630 nm and expressed as mg tyrosine/ml in compliance with a standard curve.

Examination of antibacterial activities of lactococci

The antibacterial activity of lactococi against the pathogens *Staphylococcus aureus* and *Salmonella enterica* subsp. *enterica* ser. Typhiumurium was examined using the agar diffusion method described by Nassib *et al*. (2006). *S. aureus* and *Sal*. ser. Typhimurium strains were previously isolated from dairy products and identified by El-Sharoud & Spano (2008) and Nassib *et al*. (2003), respectively. Briefly, *S. aureus* and *Sal*. ser. Typhimurium were grown overnight in nutrient broth (Oxoid, Basingstoke, UK) at 37°C. Appropriate aliquots were then applied onto the surface of nutrient agar plates (Oxoid, Basingstoke, UK) using cotton-tipped applicator. Filter paper discs (Ø 2 cm) previously immersed in cell-free filtrate (CFF) prepared from lactococci cultures was placed on each plate. To prepare CFF, an overnight culture of *Lac*. *lactis* grown in RSM (10% T.S.) was centrifuged at 5000 ×*g* for 10 min. Supernatant was filtered through 0.45 µm membrane filter (Sartorius AG, Goettingen, Germany) to prepare cell-free filtrate (CFF). Inoculated nutrient agar plates were then incubated at 35°C for 24 h. The diameter of the inhibition zone surrounding each paper disc was measured.

Statistical analysis

All presented data are the means of 3 replicates. Means were statistically compared according to the LSD test and differences were considered significant at $P<0.05$ (Little & Hills, 1996).

RESULTS AND DISCUSSION

In this study, 68 samples of Laban Rayeb and Kariesh cheese were examined for the presence of lactococci. These organisms were also isolated from two commercial LAB mesophilic starters. Thirty-seven *Lactococcus* spp. isolates were recovered (12 isolates from Laban Rayeb, 18 isolates from Kariesh cheese and 7 isolates from commercial starters) (Table 1). Lactococci isolated from Laban Rayeb and Kariesh cheese were all identified as *Lac. lactis* subsp. *lactis*, whereas isolates from commercial starters involved *Lac. lactis* subsp. *lactis*, *Lac. lactis* subsp. *cremoris* and *Lac.* spp. These results reflect the fact that Laban Rayeb is used for the preparation of Kariesh cheese, which allows the co-existence of the same LAB in both products. Commercial mesophilic LAB starters frequently contain mixtures of lactococci and this explains the isolation of diverse *Lactococcus* species from these starters. The rational behind isolating lactococci from commercial starters was to obtain strains to be compared with lactococci isolated from Labab Rayeb and Kariesh cheese. Since all lactococci recovered from the latter two products were identified as *Lac. lactis* subsp. *lactis* , only the 3 isolates of *Lac. lactis* subsp. *lactis* cultured from commercial starters were used for studying their technological characteristics and antibacterial activity compared with their counterpart isolates from Laban Rayeb and Kariesh cheese.

Table 1: Results of isolating and identifying lactococci from Laban Rayeb, Kariesh cheese and commercial starters samples

Product (Number of samples)	Isolate (Number of isolates
Laban Rayeb (28)	*Lac. lactis* subsp. *lactis* (12)
Kariesh cheese (40)	*Lac. lactis* subsp. *lactis* (18)
Commercial starters (2)	*Lac. lactis* subsp. *lactis* (3)
	Lac. lactis subsp. *cremoris* (2)
	Lac. spp. (2)
Total: 70	37

Acid production is the primary, major role of LAB starters in the preparation of fermented dairy products (Cogan *et al*., 2007). Therefore, the ability of *Lac. lactis* subsp. *lactis* isolates obtained from Laban Rayeb and Kariesh cheese to develop acidity in RSM was assessed and compared with that of *Lac. lactis* subsp. *lactis* isolates recovered from commercial starters. Data in table 2 show that the three *Lac. lactis* isolates recovered from commercial starters developed very similar titratable acidity levels in RSM within the range of 0.65- .0.66%. There were 3 *Lac. lactis* isolates recovered from Laban Rayeb (isolates 4, 5, 6) and 2 isolates from Kariesh cheese (isolates 16, 17) that developed significantly higher titratable acidity ($p<0.05$) than each of the 3 commercial starter isolates (Table 2). Whereas, there was no significant difference in acid production ($p<0.05$) between 5 isolates from Laban Rayeb (isolates 7, 8, 9, 10, 11) and 7 isolates from Kariesh cheese (isolates 18, 19, 20, 21, 22, 23, 24) and each of the 3 commercial starter isolates (Table 2). However, the remaining isolates numbered 12 through 15

and 25 through 33, which were obtained from Laban Rayeb and Kariesh cheese, respectively produced significantly lower acidity levels ($p<0.05$) than the commercial starter isolates.

Table 2: Technological properties of *Lac. Lactis* subsp. *lactis* isolates recovered from commercial starters and Laban Rayeb and Kariesh cheese*.

Isolate No.	Source	Acid production (T.A. %)	EPS production	Proteolytic activity (mg tyrosine/ml)
1	Commercial Starter	0.66 ± 0.01	**-**	0.02 ± 0.001
2	Commercial Starter	0.65 ± 0.01	**-**	0.02 ± 0.002
3	Commercial Starter	0.65 ± 0.01	**-**	0.02 ± 0.001
4	Laban Rayeb	0.82 ± 0.02	**-**	0.02 ± 0.002
5	Laban Rayeb	0.80 ± 0.01	**-**	0.02 ± 0.002
6	Laban Rayeb	0.80 ± 0.01	**-**	0.02 ± 0.001
7	Laban Rayeb	0.66 ± 0.02	**-**	0.01 ± 0.000
8	Laban Rayeb	0.67 ± 0.01	**-**	0.01 ± 0.000
9	Laban Rayeb	0.65 ± 0.01	**-**	0.01 ± 0.000
10	Laban Rayeb	0.60 ± 0.02	**-**	0.01 ± 0.000
11	Laban Rayeb	0.65 ± 0.01	**-**	0.01 ± 0.002
12	Laban Rayeb	0.50 ± 0.01	**-**	0.01 ± 0.002
13	Laban Rayeb	0.45 ± 0.01	**+**	0.01 ± 0.003
14	Laban Rayeb	0.45 ± 0.01	**+**	0.01 ± 0.003
15	Laban Rayeb	0.40 ± 0.01	**+**	0.01 ± 0.000
16	Kariesh cheese	0.82 ± 0.01	**-**	0.05 ± 0.002
17	Kariesh cheese	0.80 ± 0.01	**-**	0.04 ± 0.003
18	Kariesh cheese	0.60 ± 0.01	**-**	0.04 ± 0.002
19	Kariesh cheese	0.65 ± 0.02	**-**	0.01 ± 0.003
20	Kariesh cheese	0.66 ± 0.01	**-**	0.01 ± 0.000
21	Kariesh cheese	0.65 ± 0.01	**-**	0.01 ± 0.000
22	Kariesh cheese	0.60 ± 0.01	**-**	0.01 ± 0.000
23	Kariesh cheese	0.66 ± 0.01	**-**	0.01 ± 0.003
24	Kariesh cheese	0.60 ± 0.01	**-**	0.01 ± 0.003
25	Kariesh cheese	0.55 ± 0.02	**-**	0.01 ± 0.003
26	Kariesh cheese	0.55 ± 0.01	**-**	0.01 ± 0.003
27	Kariesh cheese	0.45 ± 0.01	**-**	0.0 ± 0.000
28	Kariesh cheese	0.45 ± 0.02	-	0.0 ± 0.000
29	Kariesh cheese	0.40 ± 0.01	-	0.0 ± 0.000
30	Kariesh cheese	0.38 ± 0.02	+	0.0 ± 0.000
31	Kariesh cheese	0.38 ± 0.01	+	0.0 ± 0.000
32	Kariesh cheese	0.40 ± 0.02	+	0.0 ± 0.000
33	Kariesh cheese	0.45 ± 0.02	+	0.0 ± 0.000

***Presented values are the means of triplicate measurements ± standard deviations**

The isolates were further examined for the production of exopolysaccharides (EPS). Table 2 shows that there were 7 out of 33 examined *Lac. lactis* isolates that had the ability to produce EPS. Interestingly, these EPS-producing isolates were among those that produced generally lower acidity levels. This observation was reported before in EPS-producing *Streptococcus thermophilus* and it was suggested that EPS-producing bacteria may spend more energy to produce EPS, which could be reflected in slower growth and slower acid production (Hassan *et al*., 1995). It was also suggested that the formation of capsules around the cells by EPS

may retard the transport of lactic acid from the cytoplasm to the surrounding environment. This may cause acid accumulation in the cytoplasm and inhibit metabolic activities leading to acid production (Hassan *et al*., 1995). The use of EPS-producing LAB starters in the preparation of dairy products has been shown to provide viscosity, stability and water-binding functions that may improve the taste and texture of fermented dairy products (De Vuyst & Degeest, 1999; Broadbent *et al*., 2003; Hassan, 2008). For this reason, EPS-producing LAB starters have been used by European processors for many years to produce a variety of fermented milks with unique properties (Cerning, 1995). However, the present results show that EPS-producing *Lac. lactis* isolates were less able to produce acidity than their EPS-nonproducing counterparts. This means that these EPS-producing *Lac. lactis* isolates will not be suitable for use in the preparation of dairy products that require the development of higher acidity.

In addition to acidity development, LAB starters also contribute to cheese ripening by synthesizing proteolytic enzymes (Cogan *et al.,* 2007). Therefore, the proteolytic activities of *Lac. lactis* isolates examined in this study were assessed as one criterion of the suitability of these isolates for use in dairy applications. As shown in table 2, there were three isolates from Laban Rayeb numbered 4, 5 & 6, whose proteolytic activities did not significantly differ ($p<0.05$) from that of each of the three *Lac. lactis* isolates recovered from commercial starters. Another 3 isolates from Kariesh cheese numbered 16, 17 & 18 showed significantly higher ($p<0.05$) proteolytic activities than each of the commercial starter strains (Table 2). The remaining isolates showed significantly lower ($p<0.05$) proteolytic activities than the commercial starter isolates, with 7 isolates numbered 27, 28, 29, 30, 31, 32 & 33 showing undetectable proteolytic activities (Table 2). This was not unexpected given that LAB are generally considered weakly proteolytic when compared with many other groups of bacteria, e.g. *Bacillus, Proteus, Pseudomonas*. Interestingly, all isolates showing proteolytic activities higher or equal to that of lacotococci isolated from commercial starters were able to adequately produce acidity. This suggests that these isolates could be used in dairy industry to replace imported commercial starters. Since some of these isolates could produce higher acidity and expressed higher proteolytic activities compared with the commercial isolates, their use in dairy industry may improve the quality of resultant dairy products.

The antibacterial activities of lactococci isolated in this study were examined against two important foodborne pathogens; *S. aureus* and *Sal.* ser. Typhimurium representing Gram-positive and Gram-negative bacteria, respectively. It could be noted from table 3 that the inhibitory effects of the examined *Lac. lactis* isolates against *S. aureus* were generally greater than their inhibitory effects against *Sal.* ser. Typhimurium. This could be attributed to the presence of the outer membrane in G-negative bacteria and its absence in G-positive bacteria. This membrane could offer protection to G-negative bacteria against inhibitory compounds produced by LAB (Boziaris & Adams, 2000).

Table 3: Antibacterial effects of *Lac. lactis* subsp. *lactis* isolates recovered from commercial starters and Laban Rayeb and Kariesh cheese*.

Isolate No.*	Source	Inhibition zone (diameter, mm) against pathogenic bacteria	
		S. aureus	***Sal*. ser. Typhimurium**
1	Commercial Starter	6 ± 0.01	4 ± 0.006
2	Commercial Starter	6 ± 0.01	4 ± 0.006
3	Commercial Starter	7 ± 0.01	4 ± 0.006
4	Laban Rayeb	10 ± 0.01	6 ± 0.01
5	Laban Rayeb	10 ± 0.02	6 ± 0.01
6	Laban Rayeb	10 ± 0.02	7 ± 0.02
7	Laban Rayeb	6 ± 0.01	4 ± 0.006
8	Laban Rayeb	6 ± 0.01	5 ± 0.01
9	Laban Rayeb	6 ± 0.01	4 ± 0.006
10	Laban Rayeb	6 ± 0.01	4 ± 0.006
11	Laban Rayeb	6 ± 0.01	4 ± 0.006
12	Laban Rayeb	3 ± 0.006	2 ± 0.008
13	Laban Rayeb	3 ± 0.01	2 ± 0.01
14	Laban Rayeb	3 ± 0.006	2 ± 0.006
15	Laban Rayeb	3 ± 0.006	2 ± 0.006
16	Kariesh cheese	10 ± 0.008	7 ± 0.01
17	Kariesh cheese	9 ± 0.01	7 ± 0.01
18	Kariesh cheese	7 ± 0.01	4 ± 0.006
19	Kariesh cheese	6 ± 0.006	4 ± 0.006
20	Kariesh cheese	7 ± 0.008	4 ± 0.01
21	Kariesh cheese	6 ± 0.007	4 ± 0.01
22	Kariesh cheese	5 ± 0.01	4 ± 0.006
23	Kariesh cheese	6 ± 0.008	4 ± 0.006
24	Kariesh cheese	6 ± 0.008	4 ± 0.01
25	Kariesh cheese	3 ± 0.01	2 ± 0.006
26	Kariesh cheese	3 ± 0.006	2 ± 0.007
27	Kariesh cheese	3 ± 0.006	2 ± 0.01
28	Kariesh cheese	4 ± 0.006	2 ± 0.007
29	Kariesh cheese	4 ± 0.01	2 ± 0.008
30	Kariesh cheese	3 ± 0.006	0
31	Kariesh cheese	3 ± 0.006	0
32	Kariesh cheese	4 ± 0.01	2 ± 0.006
33	Kariesh cheese	3 ± 0.01	2 ± 0.006

***Presented values are the means of triplicate measurements ± standard deviations**

It is also possible that some of the examined lactococci (G^+) produced bacteriocins, which are known to inhibit only closely related microorganisms (*S. aureus* in this case) (Delves-Broughton, 1990). All the 3 lactococci isolated from commercial starters showed inhibitory effects against both examined pathogenic bacteria. There were 3 isolates from Laban Rayeb numbered 4, 5 & 6 and 2 isolates from Kariesh cheese numbered 16 & 17 that showed significantly higher ($p<0.05$) inhibitory effects against *S. aureus* and *Sal*. ser. Typhimurium. These isolates produced higher acidity levels than each of the commercial starter lactococci (Table 2). Interestingly, there were no significant differences between the inhibitory effects of lactococci developing acidity levels similar to those developed by the commercial starter lactococci, e.g. isolates number 7, 8, 9, 10 and the inhibitory effect of each of

the commercial starter lactococci against *S. aureus* and *Sal*. ser. Typhimurium (Tables 2 & 3). Furthermore, the inhibitory effects of lactococci producing lower acidity levels were significantly lower ($p<0.05$) than that of the commercial starters. Together, these results show that acidity contributed a major role in the inhibition of *S. aureus* and *Sal.* ser. Typhimurium by lactococci. This is consistent with Nassib *et al*. (2006) who found that acidity was the main inhibitory factor in the antagonistic action of thermophilic LAB against *Sal.* ser. Typhimurium.

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الصفات التكنولوجية والفعالية ضد البكتريا لميكروب اللاكتوكوكس لاكتس تحت نوع لاكتس المعزول من اللبن الرايب والجبن القريش

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يعتبر ميكروب اللاكتوكوكس لاكتس تحت نوع لاكتس أحد أنواع بكتريا حامض اللاكتيك الشائع استخدامها كبادئ في صناعة المنتجات اللبنية المتخمرة، ويتم استيراد كميات هائلة منه سنوياً من الدول الغربية بما يشكل عبئاً علي الصناعات اللبنية في مصر ويعيق تقدمها، ولقد استهدفت الدراسة الحالية عزل هذا الميكروب من اللبن الرايب والجبن القريش كأحد المنتجات المتخمرة المتوافرة محلياً والتي تحتوي علي تنوع كبير نسبياً من العزلات البكتيرية، مع تعريف العزلات الناتجة ودراسة صفاتها لمعرفة أمكانية الاستفادة منها في الصناعات اللبنية، ولقد تم عزل ٣٠ مزرعة من الميكروب من اللبن الرايب والجبن القريش وتم تعريفها جميعاً وكذلك تم عزل ٣ مزارع من الميكروب من البادئات التجارية الموجودة في الأسواق بغرض مقارنتها بالعزلات المتحصل عليها من منتجات الألبان، ولقد أوضحت النتائج أن هناك بعض العزلات من اللبن الرايب والجبن القريش يمكنها إنتاج حموضة وتحليل البروتين بدرجة أعلي من عزلات البادئات التجارية (مستوي معنوية < ٠,٠٥)، كما أن بعض العزلات قامت بإنتاج السكريات الخارجية العديدة ولكن ذلك كان مرتبطاً بإنتاج حموضة أقل، ولقد وجد أن العزلات يمكنها أيضاً تثبيط ميكروبي الاستافيلوكوكس أوريس والسالمونيلا طراز سريولوجي تيفيميوريم الممرضين، وتوضح هذه النتائج مجتمعة أنه يمكن انتخاب بعض من العزلات المتحصل عليها من اللبن الرايب والجبن القريش كبديل للبادئات المستوردة في صناعة المنتجات اللبنية المتخمرة.