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CHARACTERIZATION OF *Streptococcus thermophilus* ASSOCIATED WITH EGYPTIAN DAIRY PRODUCTS

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ABSTRACT

Streptococcus thermophilus is an important industrial species that is extensively used in the preparation of fermented dairy products. The present study was designed to characterize *S. thermophilus* strains isolated from traditional Egyptian dairy products. Ninety-five samples of Kariesh cheese, Zabady, Laban Rayeb and Ras cheese were randomly collected, and examined for the presence of *S. thermophilus.* A total of 255 of lactic acid bacteria cultures could be isolated from these products and subjected to morphological and physiological examinations. Out of these isolates, 20 cultures isolated from Kariesh cheese and Laban Rayeb could be identified as *S. thermophilus* using a PCR analysis targeting the *serB* gene. These isolates were characterized on the basis of biochemical traits related to their potential use in dairy industries. This involved examining their ability to develop acidity, utilize galactose, produce exopolysaccharides and express the urease enzyme. *S. thermophilus* isolates were diverse in their ability to acidify milk, with the most of isolates developing moderate levels of acidity of 0.4% - < 0.6% after 6 h of incubation at 37°C. Isolates showed diversity in utilizing galactose and urease activity, yet the majority of them was unable to ferment galactose, or expressed urease. Only 1 isolate could produce exopolysaccharides. These results show that Kariesh cheese and Laban Rayeb could serve as a local source of *S. thermophilus* isolates of diverse technological traits that could be exploited in dairy processing.

Keywords: *Streptococcus thermophilus*, Kariesh cheese, Laban Rayeb, PCR

INTRODUCTION

Streptococcus thermophilus is a major starter component that has been heavily employed in the preparation of fermented dairy products. It is ranked the second most important species of industrial lactic acid bacteria (LAB) after *Lactococcus lactis*, with a commercial value of approximately 40 billion US dollars (Hols *et al*. 2005). Given its extensive use in the preparation of dairy products, it is estimated that humans consume more than 10^{21} cells of *S. thermophilus* every year (Hols *et al*. 2005). *S. thermophilus* is traditionally used in a combination with *Lactobacillus delbrueckii* subsp. *bulgaricus* for the preparation of yoghurt, but has been also used in the manufacture of several cheese varieties including Swiss cheese, Brick cheese, Parmesan, Provolone, Mozzarella and Asiago (Parente & Cogan 2004). *S. thermophilus* is mainly used for milk fermentation due to its ability to utilize lactose and produce lactic acid. However, it can also produce low levels of other metabolites including formate, acetoin, diacetyl, acetaldehyde and acetate that affect the flavor of dairy products (Ott *et al*. 2000). Various *S. thermophilus* strains display functional activities such as the production of

exopolysaccharides, bacteriocins and vitamins, which could improve the quality of dairy products (Lyer *et al*. 2010). Some strains have been described as potential probiotics as evidenced by their various health effects, and moderate adherence in the gastrointestinal tract. Given such diverse roles of *S. thermophilus*, it has been described as a "multifunctional" species (Lyer *et al*. 2010).

Still, a relatively high level of phenotypic diversity has been reported in *S. thermophilus* strains isolated from dairy products in Europe (Giraffa *et al*. 2011; Mora *et al*. 2002). This diversity involves metabolic characteristics that influence the performance of *S. thermophilus* during the preparation of dairy products, such as acid production, galactose utilization, urease activity and exopolysaccharides production. These characteristics could be used as a basis for selecting *S. thermophilus* strains intended for use in dairy processing. However, little is known on the patterns of these technological characteristics in *S. thermophilus* isolated from dairy products in other parts of the world including Egypt. The present study was thus designed to characterize *S. thermophilus* strains isolated from traditional Egyptian dairy products. This is to complement current knowledge on the diversity of this important industrial species, and initiate the establishment of a collection of well-characterized strains of *S. thermophilus*. This bacterial collection could serve as a local source of *S. thermophilus* strains for dairy industries, which will help save the high cost of importing dairy starters from Western countries.

MATERIALS AND METHODS

Collection of milk and dairy product samples

A total of 95 samples of dairy products were randomly collected from local markets in Mansoura city and villages in its vicinity. These samples included 36 samples of Kariesh cheese, 40 samples of Zabady, 13 samples of Laban Rayeb and 6 samples of Ras cheese.

Isolation of *Streptococcus thermophilus* from Dairy Products

Dairy product samples were serially diluted in sterilized saline solution (0.85% NaCl). Resultant dilutions were plated onto Elliker agar (BD, New Jersey, USA) and MRS agar (Oxoid, Basingstoke, UK), followed by incubation at 37°C for 48 h. Suspected colonies were picked up and maintained for identification tests.

Identification of *Streptococcus thermophilus* Isolates

Suspected isolates were subjected to morphological and biochemical examinations including Gram-staining, catalase test, milk coagulation test, growth under certain conditions of temperature, salt concentrations and pH and growth on the bile esculin agar (BEA). Prior to each test, suspected isolates were grown overnight at 37°C in MRS broth (Oxoid, Basingstoke, UK). For the catalase test, cultures were grown overnight at 37°C on MRS agar (Oxoid, Basingstoke, UK).

Gram Staining

Overnight cultures were tested for their Gram staining reactions and cell morphology as described by Pollack *et al*. (2005).

Catalase Test

Suitable amount of growth from one discrete colony of an overnight culture was transferred into a clean glass slide, followed by the addition of 1 drop of H_2O_2 (3%). Immediate bubbling (gas formation) was taken as a positive result (Macfaddin 1977).

Milk Coagulation

Overnight cultures were inoculated at 1% (v/v) into sterilized reconstituted skim milk (10% total solids), followed by incubation at 37°C. Cultures were observed for milk coagulation every 30 minutes for up to 6 h.

Growth at 45°C and 10°C

Overnight cultures were inoculated 1% (v/v) into MRS broth followed by incubation at 45°C for 48 h or 10°C for 2 weeks. Cultures were observed for growth under these conditions (Sharpe 1979).

Growth in 4% and 6.5% NaCl

Overnight cultures were inoculated at 1% (v/v) into MRS broth containing 4% or 6.5% NaCl, followed by incubation at 37°C (Abd El-Malek and Gibson 1948). Cell growth was observed after 48 h of incubation.

Growth at pH 9.6

Overnight cultures were inoculated at 1% (v/v) into MRS broth adjusted to pH 9.6, followed by incubation at 37°C (Sharpe 1979). Cell growth was observed after 48 h of incubation.

Growth on the BEA medium

One hundred microliters of an overnight culture was spread onto bile esculin agar (BEA) (BD, New Jersey, USA), followed by incubation at 37°C for 24 h. BEA plates were then examined for the development of black colonies.

Polymerase Chain Reaction (PCR) Identification of Potential *Streptococcus thermophilus* Isolates

Potential *S. thermophilus* isolates that displayed typical results in the above examinations were further identified using the PCR analysis as described by El-Sharoud *et al*. (2011). Cultures were grown overnight at 37°C on M17 agar (Oxoid, Basingstoke, UK) containing lactose at a final concentration of 1.0%. One discrete bacterial colony from each M17 agar plate was suspended in 50 µl of TES (10 mM Tris-HCl, 1 mM EDTA, 25% sucrose), and DNA was extracted by cell lysis at a high temperature of 95°C for 10 min followed by cooling at 4°C for 15 min using the Primus 25 advanced thermocycler (peQLab, Wilmington, Delware, USA). A PCR reaction mixture of 50 µl was formulated using 5 µl bacterial DNA, 25 µl mater mix (peqGOLD PCR-MasterMix, peQLab, Wilmington, Delware, USA), 2 µl of forward primer (GGTCCAAGAAGAAGTAATTGA), 2 µl reverse primer (GACCTTATACAAATCTGGTT), and 16 µl water. Primers were designed to target the *serB* gene encoding phosphoserine phosphatase in *S. thermophilus* and were synthesized by Applied Biosystems (Applied Biosystems, Foster City, CA, USA). PCR reactions were conducted in the Primus 25 advanced thermocycler using the following cycling parameters: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min. Resultant PCR amplicons were separated by gel electrophoresis using 1% agarose gel in TBE buffer (AppliChem, Darmstadt,

Germany). Gel was visualized using UV transilluminator (Vilber lourmat, France) and photo was captured using a gel documentation system.

Technological and Biochemical Characterization of *Streptococcus thermophilus* Isolates

Identified *S. thermophilus* isolates were characterized for technological and biochemical traits related to their potential use in dairy processing as follows:

Acidity Development

Overnight cultures of *S. thermophilus* isolates grown in sterilized reconstituted skim milk containing 10% total solids (RSM-10%) were inoculated at 1% (v/v) into new sterilized amount of RSM-10% followed by incubation at 37°C for 6 h. Samples were taken at an interval of 1 h for determining titratable acidity % (T.A%).

Galactose Utilization

Overnight cultures of *S. thermophilus* isolates grown in MRS broth were inoculated at 1% (v/v) into basal sugar medium (BSM) containing chlorophenol red (0.4% w/v) and supplemented with filter-sterilized galactose (0.2% w/v), followed by incubation at 37°C for 24 h (de Man *et al*. 1960). The utilization of galactose by *S. thermophilus* isolates was indicated by a change of the indicator color into yellow.

Urease Activity

Urease activity of *S. thermophilus* isolates was assessed using the method reported by Lanyi (1987). A loopful of an overnight culture grown in MRS broth was inoculated into a compound solution containing one volume of solution A and 19 volumes of solution B. Solution A was prepared by dissolving 2 g urea in 2 ml ethanol, followed by addition of 4 ml sterilized water. Solution B consisted of KH_2PO_4 (1 g l^{-1}), K_2HPO_4 (1 g l^{-1}), NaCl, (5 g l^{-1}), and phenol red (20 µg ml^{-1}). Inoculated suspension was incubated at 37°C for 1–2 h, where the development of red-violet color indicated positive urease activity.

Exopolysaccharide (EPS) Production

S. thermophilus isolates grown overnight at 37°C in sterilized RSM-10% were inoculated at 2% (v/v) into new amounts of sterilized RSM-10%, followed by incubation at 37°C for 24 h. Resultant cultures were gently stirred for 5-7 times with a spoon. The formation of stick, continued threads on withdrawing samples of coagulated milk by the spoon was taken as an indicator of EPS production.

RESULTS AND DISCUSSION

Isolation of *Streptococcus thermophilus* from Traditional Egyptian Dairy Products

Ninety five (95) samples of traditional Egyptian dairy products were randomly collected from local markets in Mansoura City and villages in its vicinity. These samples included 6 samples of Ras cheese, 40 samples of Zabady, 13 samples of Laban Rayeb and 36 samples of Kariesh (Table 1). Samples were serially diluted and plated onto Elliker agar and MRS agar.

Suspected lactic acid bacteria (LAB) colonies were picked up from agar plates and subjected to preliminary identification involving Gram-staining, catalase test, and milk coagulation test.

Table 1 shows the varieties and numbers of examined dairy samples and the numbers of potential LAB isolates recovered from each product. Gram-positive and catalase-negative isolates that could coagulate milk were considered as potential lactic acid bacteria (LAB). Microscopic examination of these isolates showed that out of 255 potential LAB cultures, 222 isolates were cocci and 33 isolates were lactobacilli. Since the focus of this study was to isolate *S. thermophilus,* only the cocci isolates were maintained for further identification.

As shown in table 2, 20 out of 222 cocci could not grow at 10°C, pH 9.6 or in the presence of 4% or 6.5% NaCl, but could grow at 45°C. These isolates did not produce colonies on the bile esculin agar (BEA).

Table 1: Isolation and Preliminary Identification of Lactic Acid Bacteria from Traditional Egyptian Dairy Products

Samples	No. of Samples	No. of Potential LAB Isolates	Potential LAB Isolates	
			Cocci	Lactobacilli
Ras cheese	6	12	7	5
Kariesh cheese	36	132	129	3
Zabady	40	58	43	15
Laban Rayeb	13	53	43	10
Total	95	255	222	33

Table 2: Further Identification of Potential LAB Isolates Recovered from Traditional Egyptian Dairy Products.

Potential LAB Isolates (No. of Isolates)	Growth at 10°C	Growth at 45°C	Growth at pH 9.6	Growth in 4.0 % NaCl	Growth in 6.5% NaCl	Growth on BEA medium	Results of Identification (No. of Isolates)
Cocci (222)	-	+	-	-	-	-	*Streptococcus* spp(20)
	+	+	+	+	+	+	*Enterococcus* spp (202)

Together, these results suggested that these 20 isolates belonged to the *Streptococcus* genus (Sharpe 1979 and Hardie & Whiley 1995). However, the other 202 cocci could grow at 10°C, 45°C, pH 9.6 and in the presence of 4 % or 6.5% NaCl. These cultures could also grow on the BEA medium forming black colonies. This suggested the belonging of these 202 cocci to the *Enterococcus* species (Sharpe 1979 and Hardie & Whiley 1995).

These results show that *Enterococcus* spp. are more prevalent in Egyptian dairy products than *Streptococcus* spp. This could be attributed to the ability of *Enterococcus* strains to tolerate adverse environmental conditions of low pH and high salt levels existing in traditional Egyptian dairy products (Jokovic *et al.* 2008).

Polymerase Chain Reaction (PCR) Identification of *Streptococcus* spp. isolates

In order to screen out *Streptococcus thermophilus* strains, the 20 potential *Streptococcus* spp. isolates were subjected to a PCR analysis targeting the *serB* gene that encodes the phosphoserine phophatase in *S. thermophilus* (Bolotin *et al*. 2004; Hols *et al*. 2005). Figures 1 A, B & C show photos of agarose gel resulted from PCR analysis of the 20 potential *Streptococcus* spp. These gel photos present the separation of the *serB* amplicon from 16 isolates recovered from Kariesh cheese (Fig. 1 A & B), and 4 isolates recovered from Laban Rayeb (Fig. 1 C). This confirmed the belonging of these 20 isolates to the *S. thermophilus* species.

Figure 1: PCR Identification of 20 *Streptococcus* isolates recovered from traditional dairy products. These isolates included 16

cultures recovered from Kariesh cheese (A & B) and 4 cultures from Laban Rayeb (C).

The above results show that traditional Egyptian dairy products examined in this study could contain both LAB cocci and lactobacilli. This is consistent with previous findings reported by Ayad *et al*. (2004 & 2006), who isolated several LAB genera including *Lactococcus*, *Lactobacillus*, *Enterococcus*, *Streptococcus* and *Pediococcus* from Domiatti cheese, Ras cheese, Mish, Zabady and Laban Rayeb. The occurrence of *S. thermophilus* in traditional Egyptian dairy products including Zabady and Mish was also reported by El-Baradei *et al*. (2004 & 2008).

Characterization of *Streptococcus thermophilus* Strains Isolated from Traditional Egyptian Dairy Products

Streptococcus thermophilus isolates recovered from traditional dairy products were characterized in terms of biochemical properties related to their potential use in dairy industries. This involved examining the ability of these isolates to develop acidity, utilize galactose, produce exopolysaccharides and express the urease enzyme.

Acidity Development

S. thermophilus is frequently used as a starter culture for fermenting lactose and developing acidity during the preparation of fermented dairy products. Therefore, the ability of *S. thermophilus* strains to develop acidity in milk is a major criterion in assessing their potential use in dairy industries. Table 3 shows acidity development by 20 *S. thermophilus* isolates recovered from traditional Egyptian dairy products over 6 h of incubation at 37°C in sterilized reconstituted skim milk. Isolates were variable in their ability to develop acidity in milk that they could be categorized into three groups. The first group involved isolates, whose acid production was relatively slow being < 0.4% after 6 h. This group included 4 isolates designated 17, 37, 70 and 98. The second group contained isolates that produced moderate acidity levels of 0.4% - < 0.6% after 6 h. This group included the largest number of isolates (12 isolates) designated 2, 10, 13, 34, 40, 50, 51, 52, 59, 65, 76 and 101. The third group involved isolates of a strong ability to produce acidity, as they developed acidity levels of ≥ 0.6% after 6 h.

This group encompassed 4 isolates designated 16, 100, 109 and 198. It could be concluded that while *S. thermophilus* isolates were diverse in their ability to acidify milk, most of them developed moderate levels of acidity.

Galactose Utilization

As shown in table 3, 8 out of 20 isolates of *S. thermophilus* (40% of total isolates) could ferment galactose, while 12 isolates (60% of total isolates) were unable to utilize this monosaccharide. This is similar to the finding of Mora *et al*. (2002), who reported that 75% of 44 *S. thermophilus* strains were unable to utilize galactose, whereas 24% of those strains could ferment it. Taken together, this confirms that galactose utilization by *S. thermophilus* is a strain-dependent trait. The use of *S. thermophilus* strains unable to ferment galactose as starters for cheese making was implicated in browning problems when cheese was processed or cooked at high temperatures (Olson 1983). Residual galactose in cheese could also serve as a substrate for heterofermentative microorganisms in cheese, which results in

carbon dioxide production and textural defects (Tinson *et al*. 1982; Hutkins *et al*. 1986). Therefore, galactose-utilizing strains of *S. thermophilus* are preferred starter cultures for dairy industries. The present work introduces 8 galactose-utilizing *S. thermophilus* isolates cultured from local dairy products.

Table 3: Technological and Biochemical Traits of *Streptococcus thermophilus* Isolated Cultured from Traditional Egyptian Dairy Products

Isolate No.	Source	Titratable Acidity% (TA%)							Galactose Utilization	Urease Activity	EPS Production
		0	1	2	3	4	5	6			
2	KC*	0.18	0.25	0.29	0.33	0.36	0.39	0.45	**+**	-	**-**
10	KC	0.18	0.20	0.23	0.33	0.39	**0.5**	0.59	**+**	-	**-**
13	LR**	0.18	0.25	0.30	0.35	0.45	0.5	0.55	**-**	+	**-**
16	KC	0.18	0.22	0.25	0.38	0.43	**0.55**	0.60	**+**	-	**-**
17	KC	0.18	0.21	0.24	0.25	0.31	0.35	0.38	**+**	+	**-**
34	KC	0.18	0.23	0.26	0.37	**0.45**	0.50	0.53	**-**	W***	**-**
37	KC	0.18	0.27	0.29	0.32	0.35	0.37	0.39	**-**	W	**-**
40	KC	0.18	0.25	0.30	0.33	0.36	0.40	0.46	**-**	+	**-**
50	KC	0.18	0.26	0.29	0.31	0.35	0.41	0.44	**-**	+	**-**
51	KC	0.18	0.20	0.23	0.25	0.30	0.35	0.43	**-**	+	**-**
52	KC	0.18	0.20	0.25	0.32	0.40	0.46	0.50	**-**	+	**-**
59	KC	0.18	0.30	0.31	0.34	0.38	0.41	0.42	**-**	-	**-**
65	KC	0.18	0.29	0.31	0.33	0.35	0.40	0.42	**+**	W	**-**
70	KC	0.18	0.24	0.26	0.3	0.31	0.35	0.37	**+**	-	**-**
76	LR	0.18	0.21	0.28	0.35	0.45	**0.51**	0.57	**+**	W	**-**
98	KC	0.18	0.19	0.25	0.28	0.30	0.33	**0.37**	**-**	+	**-**
100	LR	0.18	0.28	0.32	0.45	**0.64**	0.7	0.72	**-**	+	**-**
101	LR	0.18	0.22	0.27	0.31	0.37	0.43	**0.46**	**-**	+	**-**
109	KC	0.18	0.26	0.30	0.39	0.5	**0.65**	0.72	**-**	+	**+**
198	KC	0.18	0.26	0.40	0.5	0.58	0.64	0.67	**+**	-	**-**

***KC: Kariesh cheese, ** LR: Laban Rayeb, *** W: Weak.**

Urease Activity

Results presented in table 3 show that 10 out of 20 isolates (50%) of *S. thermophilus* designated 13, 17, 40, 50, 51, 52, 98, 100, 101, and 109 could express urease activity. Whereas, 6 isolates (30% of total isolates) designated 2, 10, 16, 59, 70, and 198 were urease-negative. The remaining 4 isolates showed weak urease activity. These results are consistent with those of Mora *et al*. (2002), who examined 44 isolates of *S. thermophilus* and reported that the majority of these isolates were urease-positive.

S. thermophilus is the only species among lactic acid bacteria that could display significant urease activity (Hols *et al*. 2005). The urease enzyme induces the hydrolysis of urea that exists naturally in milk at concentrations of 0.2 - 0.4 g l^{-1}. This leads to the formation of ammonia that affects flavor and acidification rates during dairy processes (Tinson *et al*. 1982; Monnet *et al*. 2004, Pernaud *et al*. 2004). It was suggested that the use of urease-positive strains of *S. thermophilus* could be associated with slower

acidification rates compared with the urease-negative counterpart strains (Monnet *et al*. 2004, Pernoud *et al*. 2004). However, our results show that there was no correlation between the rate of acid production and urease activity by *S. thermophilus* isolates. For instance, two urease-positive isolates designated 100 and 109 had strong ability to develop acidity in milk, whereas the urease-negative isolate 70 produced limited level of acidity (Table 3). This suggests that other factors beside urease activity can influence acid production by *S. thermophilus.* Marino *et al*. (2003) reported that the rate of milk acidification by *S. thermophilus* is strain-dependent and could be influenced by several factors including lactose–galactose metabolism, proteolytic system and ureolytic activity.

Exopolysaccharides (EPS) Production

S. thermophilus isolates were examined for their ability to produce exopolysaccharides (EPS) in milk by inoculating sterilized reconstituted skim milk with these isolates, followed by incubation at 37°C for 24 h. Resultant cultures were gently stirred for 5-7 times with a spoon, and examined for the formation of stick, continued threads of coagulated milk. As shown in table 3, only 1 isolate of *S. thermophilus* designated 109 was able to produce EPS in this testing.

S. thermophilus strains have been frequently reported as EPS-producing organisms. Mora *et al*. (2002) found that 50% of 44 *S. thermophilus* strains isolated from dairy products in Europe were able to produce exopolysaccharides. Vaningelgem *et al*. (2004) could also characterize EPS-producing *S. thermophilus* strains isolated from European dairy products. The incorporation of EPS-producing LAB in dairy products has been found to provide viscosity, stability and water-binding functions that improve the taste and texture of fermented dairy products (De Vuyst and Degeest 1999). Therefore, EPS-producing LAB starters have been extensively utilized in dairy industries over last decades.

In conclusion, the above results show that Kariesh cheese and Laban Rayeb contain *S. thermophilus* isolates of diverse technological and biochemical traits. A number of these isolates display traits of high acid production, galactose utilization, urease activity and EPS synthesis which could improve the manufacture and quality of dairy products. Together with an appropriate safety assessment of these isolates, they could replace *S. thermophilus* imported from Western countries.

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دراسة صفات ميكروب الاستربتوكوكس ثرموفيلس المعزول من منتجات الألبان المصرية التقليدية.

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ميكروب الاستربتوكوكس ثرموفيلس هو أحد الميكروبات الصناعية الهامة التي يتم استخدامها بكثرة في صناعة منتجات الألبان المتخمرة. وقد استهدفت هذه الدراسة عزل مزارع من هذا الميكروب من منتجات الألبان المصرية التقليدية مع دراسة صفات فسيولوجية معينة في هذه المزارع لها علاقة بإستخدام الميكروب في الصناعات اللبنية. تم تجميع ٩٥ عينة من الجبن القريش والزبادي واللبن الرايب والجبن الرأس، وأمكن عزل ٢٥٥ مزرعة من بكتريا حامض اللاكتيك من تلك العينات مع تعريف ٢٠ مزرعة منها - مصدرها الجبن القريش واللبن الرايب - علي أنها تنتمي إلي نوع الاستربتوكوكس ثرموفيلس وذلك بإستخدام تحليل "تفاعل السلسلة المتبلمر" (PCR). كذلك فقد تم دراسة قدرة هذه المزارع علي إنتاج الحموضة، وتخمير الجالاكتوز، وإنتاج الإنزيم المحلل لليوريا، وإنتاج السكريات الخارجية العديدة. أظهرت النتائج وجود تنوع في هذه الصفات فيما بين مزارع الاستربتوكوكس ثرموفيلس المختبرة. ولكن أغلب المزارع أنتجت مستويات معتدلة من الحموضة، ولم تتمكن من تخمير الجالاكتوز، وأستطاعت إنتاج الإنزيم المحلل لليوريا. وأظهرت مزرعة واحدة القدرة علي إنتاج السكريات الخارجية العديدة. تشير هذه النتائج إلي إمكانية إستخدام الجبن القريش واللبن الرايب كمصادر محلية لمزارع استربتوكوكس ثرموفيلس يمكن الاستفادة منها في الصناعات اللبنية.

قام بتحكيم البحث

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