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Incidence of *Enterococcus termitis* in Cheese Whey

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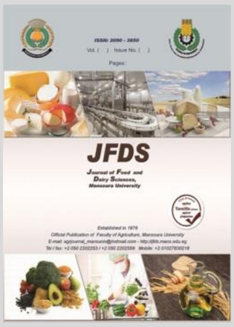

ABSTRACT

Enterococcus is one of the most predominant genus in milk, dairy products, and dairy effluents. The present study aimed to study the presence of *Enterococcus termitis* in dairy effluent (cheese whey) samples which were collected from different Egyptian dairy plants. Samples were surface plated on plates of TSA medium using miles-misra plating method. 13 isolates out of 86 bacterial isolates were suspected to belong to the genus Enterococcus. Suspected isolates were examined morphologically. Additionally, several biochemical tests like catalase, starch hydrolysis, citrate utilization, caseinase, lysine decarboxylase, lysine deaminase, H_2S production, Esculine hydrolysis and growth on MSA medium were applied on the isolates. Moreover, the suspected isolates were examined for the ability to ferment Lactose, Glucose, Galactose, Fructose, dextrose, Mannose, Ribose, Xylose, and Manitol. Results of morphological and biochemical testes indicated that the examined isolates are *Enterococcus termitis*. Results of 16s RNA sequencing indicated that the examined strains are *Enterococcus termitis* strain LMG 8895.

Keywords: cheese whey, dairy plants, morphological and biochemical characteristics, phylogenetic tree, *Enterococcus termitis*.

INTRODUCTION

Enterococci are Gram positive cocci occurring singly, in pairs, in short chains or in groups. They are air-tolerant, catalase-negative cocci relating to the lactic-acid bacteria (LAB) group. They vary from other Gram-positive, catalase-negative cocci in several phenotypic characteristics, like their ability to grow in moderately stressed conditions as growth between 10 and 45 °C, growth in hyper saline solutions, growth at pH 9.6, growth in 40% bile, and recover their viability after being heated to 60 °C for 30 min (Hardie and Whiley, 1997; Morrison *et al*., 1997; Holzapfel and Wood, 2014).

The genus Enterococcus considered the most frequent bacterial groups in various ecosystems. Due to its adaptability to adverse environmental conditions, it is frequently isolated from several natural sources such as soil, water, plants and gastrointestinal (GI) tract of mammals (Lebreton, Willems, and Gilmore 2014) In addition, it found to be one of the most frequent bacterial communities of many spontaneously fermented, traditional cheese types because it contributes to the fermentation and ripening process. Many Enterococcus species which isolated from cheese types participate in the development of the particular sensory characteristics of these types of cheese. This due to the ability of Enterococcus species to secrete proteolytic and lipolytic enzymes which hydrolyze protein and milk fat partially resulting in the production of flavor components such as acetaldehyde, acetoin, and diacetyl (Fuka *et al*., 2017; Maria *et al*., 2015; Jamaly *et al*., 2010; Giménez-Pereira, 2005). As native members of the GI tract, Enterococci can have a beneficial effect on human health by balancing gut microbiota; therefore, they may also act as probiotic bacteria. (Foulquie Moreno *et al*., 2006).

Furthermore, some Enterococcus species of dairy origin have been reported to produce bacteriocins that inhibit food spoilage and the development of pathogenic bacteria, such as *Listeria monocytogenes*, *Staphylococcus aureus* and *Vibrio cholerae* (Giraffa, 2003). Additionally, some species of Enterococcus are used as probiotics to treat diarrhea and improve immunity (Franz *et al*., 2011).

In contrast to these positive roles, some species of Enterococcus were reported to have pathogenic properties to humans due to their having specific virulence factors. The most Enterococcus species which related to human infections are *E. faecalis* and *E. faecium*. They were reported to occurring many diagnostic infections including urinary tract infection, bacteremia, endocarditis, neonatal infection and infection of the central nervous system (Moellering, 1992; O'Driscoll and Crank, 2015). Moreover, due to the widespread use of antibiotics, Enterococcus species acquired resistance to numerous antibiotics, particularly, vancomycin, teicoplanin, ampicillin, tetracycline, and cephalothin. So, some Enterococcus species are emerging as an important cause of multidrug resistance and hospital-acquired infection (Lebreton *et al*., 2014).

Due to the variety of Enterococcus species and their importance in food, feed, environmental and clinical samples, the detection and enumeration of Enterococci have become an important concern in current research activities. So this study aim to the detection of some Enterococcus species in different types of cheese whey.

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MATERIALS AND METHODS

Collection of samples:

Twenty one cheese whey samples were collected from different Dairy plants which located in Dakahlia and Damietta governorates. The type of cheese whey samples are non-salted Karish cheese whey, fresh white cheese whey, pickled white cheese whey, and Rass cheese whey. Samples were transferred to the laboratory immediately and stored at 4°C up to be used.

Isolation of bacterial strains from cheese whey:

Samples were thoroughly shacked to have a representative sample. Samples were serially diluted in sodium chloride saline (0.85% NaCl). Then, 100 µL from each dilution were streaked on tryptone soya agar (TSA) plates (Oxoid, Basingstoke, UK). The inoculated plates were incubated at 37ºC for 24 hour. Suspected colonies were picked and maintained on TSA slants at 4ºC for further examinations.

Phenotypic Identification of suspected *Enterococcus termitis* isolates:

Isolates were examined for their colony characters such as shape, colour, margin, elevation and opacity. In addition, they were examined for their morphological characters such as Gram reaction, shape and arrangement of cells.

Biochemical Identification of suspected *Enterococcus termitis* isolates:

Isolates were applied to biochemical tests including: catalase test, starch hydrolysis test, Caseinase test, lipase test, MR test, Citrate test, Kliger iron agar (KIA) test, lysine iron agar (LIA) test, Esculin hydrolysis test, growth on manitol salt agar (MSA) medium. Besides, sugars fermentation test like Ribose, Mannose, Manitol, Glucose, Galactose, Lactose, fructose and xylose. All biochemical testes were carried out according to Bergey's Manual of systematic Bacteriology (second edition, volume three; 2009).

PCR Identification of potential *Enterococcus termitis* isolates

1) Preparing of bacterial culture

Potential *Enterococcus termitis* isolates were grown on plates of tryptone soya agar (TSA) at 37C for 24h. An individual colony of each isolate was picked up and inoculated separately into 10 ml sterile tryptone soya broth (TSB). The inoculated TSB medium tubes were incubated at 37 C for 24 h. one milliliter of each activated *Enterococcus termitis* culture was transferred to 250 ml flask containing 100 ml sterile TSB medium. The incubation was conducted at 37C for 24h. The final activated cultures of *Enterococcus termitis* were used in the following step of PCR identification.

2) DNA Extraction procedure

200 µL of overnight *Enterococcus termitis* culture broth was transferred into microcentrifuge tube. Then, 95 µL water , 95 µL solid tissue buffer (blue) and 10 µL proteinase K were added to the culture broth into the microcentrifuge tube. The tubes were mixed thoroughly and then incubated at 55ºC for 2 hours. Mix the tubes thoroughly and centrifugation at 12000 x g for 1 minute. Transfer 300 µL of each aqueous supernatant to a clean tube. Add 600 µL Genomic Binding Buffer and mix thoroughly. Transfer the mixture to a Zymo-Spin™ IIC-XL Column in a Collection Tube. Centrifuge (≥ 12,000 x g) for 1 minute. Discard the collection tube with the flow through. Add 400 µL DNA Pre-Wash Buffer to the column in a new Collection Tube and centrifuge at 12000 xg for 1 minute. After that, Add 700 µL g-DNA Wash Buffer and centrifuge at 12000 xg for 1 minute. Empty the Collection Tube. Add 200 µL g-DNA Wash Buffer and centrifuge at 12000 xg for 1 minute. Discard the collection tube. Finally, Add 30 µL elution buffer incubate for 5 minutes, and then centrifuge at 12000 xg for 1 minute.

3) PCR conditions

A- PCR reaction set-up:

PCR reaction mixture of 50 µl was formulated using 8 µL bacterial DNA Template, 25 µL MyTaq Red mater mix, 1 µl (20 Pico mol) Forward Primers, 1 µl (20 Pico mol) Reverse Primers, and 15 µL Nuclease Free Water.

B- Thermal Cycler Condition:

Stages	Temperature	Time	Cycles
Initial denaturation	94 °C	6 minutes	
Denaturation	94 °C	45 seconds	35 Cycles
Annealing	56 °C	45 seconds (35 cycles)	
Extension	72 °C	1 minute	
Final Extension	72 °C	5 minutes	

Resultant PCR amplicons were separated by gel electrophoresis using 1% agarose gel in TBE buffer. Gel was visualized using UV transiluminator and photo was captured using a gel documentation system.

RESULTS AND DISCUSSION

Isolation and phenotypic characteristics of suspected *Enterococcus termitis* from cheese whey

Among 86 bacterial isolates that isolated from the different cheese whey samples, 13 isolates showed the typical morphological characteristics of *Enterococcus termitis* (Table 1).

Biochemical Identification tests of the suspected *Enterococcus termitis* isolates:

Tables 2 and 3 illustrated the biochemical reactions of the examined bacterial isolates in several biochemical tests like catalase test, Starch hydrolysis test, citrate utilization test, Caseinase test, Kliger iron agar (KIA) test, lysine iron agar (LIA) test, Esculin hydrolysis test and growth on medium containing 7% NaCl (Manitol salt agar, MSA) test. As shown in table 2, the examined isolates have the ability to hydrolyze casein but have not the ability to hydrolyze starch. Additionally, they show a negative reaction in each of catalase and citrate tests.

Table 3 illustrated that the isolates are lactose and dextrose fermenters (yellow Slant & yellow Butt) without production of neither gas nor H_2S in KIA test. Isolates couldn't produce lysine deaminase (Red slant) nor lysine decarboxylase (purple Butt) in LIA test. However, isolates have the ability to grow in the presence of bile salts and to hydrolyze esculine (Black color) through growing onto Bile Esculine agar medium. Additionally, its ability to tolerate and grow in the presences of 7.5% NaCl in MSA medium with showing weak reaction (reddish yellow) as a manitol fermenters in the MSA test.

Sugars fermentation Test:

The suspected isolates were examined for their ability to ferment several sugars. As shown in table 4, isolates are very good fermenters of each of lactose, glucose, galactose, fructose, ribose, xylose, and mannose but shown very weak reaction as manitol fermenters. Concerning of gas not produced during the fermentation of all examined sugars.

Table 1. morphological characteristics of the suspected *Enterococcus termitis* isolates recovered from cheese whey samples

No	code of isolate	color	Shape	margin	opacity	elevation	consistency	Gram reaction	Spore formation	Shape of cell	Cell Arrangement
1	1	white	punctiform	entire	Opaque	flat	smooth	G^+	-	cocci	In pairsss or small groups
2	2	white	punctiform	entire	Opaque	flat	smooth	G^+	-	cocci	In pairsss or small groups
3	9	white	punctiform	entire	Opaque	flat	smooth	G^+	-	cocci	In pairsss or small groups
4	20	white	punctiform	entire	Opaque	flat	smooth	G^+	-	cocci	In pairsss or small groups
5	21	white	punctiform	entire	Opaque	flat	smooth	G^+	-	cocci	In pairsss or small groups
6	22	white	punctiform	entire	Opaque	flat	smooth	G^+	-	cocci	In pairsss or small groups
7	23	white	punctiform	entire	Opaque	flat	smooth	G^+	-	cocci	In pairs or small groups
8	26	white	punctiform	entire	Opaque	flat	smooth	G^+	-	cocci	In pairsss or small groups
9	27	white	punctiform	entire	Opaque	flat	smooth	G^+	-	cocci	In pairsss or small groups
10	29	white	punctiform	entire	Opaque	flat	smooth	G^+	-	cocci	In pairsss or small groups
11	32	white	punctiform	entire	Opaque	flat	smooth	G^+	-	cocci	In pairsss or small groups
12	38	white	punctiform	entire	Opaque	flat	smooth	G^+	-	cocci	In pairsss or small groups
13	54	white	punctiform	entire	Opaque	flat	smooth	G^+	-	cocci	In pairsss or small groups

Table 2. Biochemical characteristics of the suspected *Enterococcus termitis* isolates recovered from cheese whey samples

Code of isolates	catalase	citrate	starch	Caseinase
1	-	-	-	+
2	-	-	-	+
9	-	-	-	+
20	-	-	-	+
21	-	-	-	+
22	-	-	-	+
23	-	-	-	+
26	-	-	-	+
27	-	-	-	+
29	-	-	-	+
32	-	-	-	+
38	-	-	-	+
54	-	-	-	+

Table 3. Biochemical characteristics of the suspected *Enterococcus termitis* isolates recovered from cheese whey samples.

Code of isolates	KIA				LIA				MSA		BEA	
	'Yellow slant' Lactose fer.	'Yellow Butt' Dextrose fer.	Gas	H_2s	'Red Slant' lysine deaminase	'purple Butt' Lysine decarboxylase	H_2s	Gas	growth	Manitol fer.	growth	Esculin fer.
1	(+) yellow	(+) yellow	-	-	(-) yellow	(-) yellow	-	-	good growth	(+) reddish yellow	good growth	Black
2	(+) yellow	(+) yellow	-	-	(-) yellow	(-) yellow	-	-	good growth	(+) reddish yellow	good growth	Black
9	(+) yellow	(+) yellow	-	-	(-) yellow	(-) yellow	-	-	good growth	(+) reddish yellow	good growth	Black
20	(+) yellow	(+) yellow	-	-	(-) yellow	(-) yellow	-	-	good growth	(+) reddish yellow	good growth	Black
21	(+) yellow	(+) yellow	-	-	(-) yellow	(-) yellow	-	-	good growth	(+) reddish yellow	good growth	Black
22	(+) yellow	(+) yellow	-	-	(-) yellow	(-) yellow	-	-	good growth	(+) reddish yellow	good growth	Black
23	(+) yellow	(+) yellow	-	-	(-) yellow	(-) yellow	-	-	good growth	(+) reddish yellow	good growth	Black
26	(+) yellow	(+) yellow	-	-	(-) yellow	(-) yellow	-	-	good growth	(+) reddish yellow	good growth	Black
27	(+) yellow	(+) yellow	-	-	(-) yellow	(-) yellow	-	-	good growth	(+) reddish yellow	good growth	Black
29	(+) yellow	(+) yellow	-	-	(-) yellow	(-) yellow	-	-	good growth	(+) reddish yellow	good growth	Black
32	(+) yellow	(+) yellow	-	-	(-) yellow	(-) yellow	-	-	good growth	(+) reddish yellow	good growth	Black
38	(+) yellow	(+) yellow	-	-	(-) yellow	(-) yellow	-	-	good growth	(+) reddish yellow	good growth	Black
54	(+) yellow	(+) yellow	-	-	(-) yellow	(-) yellow	-	-	good growth	(+) reddish yellow	good growth	Black

Table 4. sugars Fermentation by the suspected *Enterococcus termitis* isolates recovered from cheese whey samples.

Code of isolates	Glucose		Lactose		Galactose		Ribose		Xylose		Mannose		Fructose		Manitol	
	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas
1	+	-	+	-	+	-	+	-	+	-	+	-	+	-	(+) v.weak	-
2	+	-	+	-	+	-	+	-	+	-	+	-	+	-	(+) v.weak	-
9	+	-	+	-	+	-	+	-	+	-	+	-	+	-	(+) v.weak	-
20	+	-	+	-	+	-	+	-	+	-	+	-	+	-	(+) v.weak	-
21	+	-	+	-	+	-	+	-	+	-	+	-	+	-	(+) v.weak	-
22	+	-	+	-	+	-	+	-	+	-	+	-	+	-	(+) v.weak	-
23	+	-	+	-	+	-	+	-	+	-	+	-	+	-	(+) v.weak	-
26	+	-	+	-	+	-	+	-	+	-	+	-	+	-	(+) v.weak	-
27	+	-	+	-	+	-	+	-	+	-	+	-	+	-	(+) v.weak	-
29	+	-	+	-	+	-	+	-	+	-	+	-	+	-	(+) v.weak	-
32	+	-	+	-	+	-	+	-	+	-	+	-	+	-	(+) v.weak	-
38	+	-	+	-	+	-	+	-	+	-	+	-	+	-	(+) v.weak	-
54	+	-	+	-	+	-	+	-	+	-	+	-	+	-	(+) v.weak	-

Bergey's Manual and _ABIS_ identification system

By comparing the results of the examined isolates in the above conducted morphological and biochemical tests with the *Bergey's* Manual of systematic Bacteriology (second edition, volume three; 2009) and ABIS Encyclopedia online bacterial identification system, it illustrated that the examined bacterial isolates belonged to *Enterococcus termitis.*

Polymerase Chain Reaction (PCR) Identification of suspected *Enterococcus termitis* isolates recovered from cheese whey samples.

For further identification, the potential *Enterococcus termitis* isolates were identified by PCR followed by RNA sequencing. Results show that isolates are *Enterococcus termitis* strain LMG 8895.(Fig.1.)

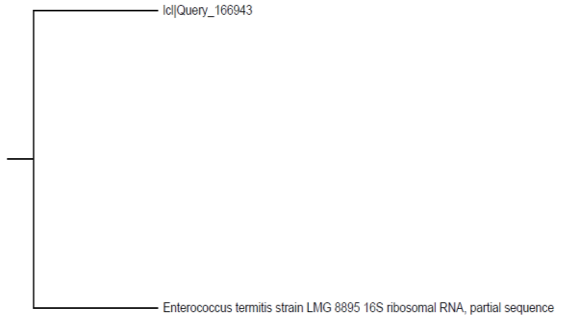

Fig.1. The phylogenetic tree 16s ribosomal RNA of *Ent. termitis* isolates which recovered from cheese whey samples.

The obtained results agree with Mazzucotelli *et al*. (2013) who studied the isolation of lipolytic and proteolytic bacteria from six different agro-industrial by-products and wastes including cheese whey. *Enterococcus* was one of the Predominate bacterial genus in the examined samples. The isolated strains were genetically identified as *Enterococcus faecalis* strain HN-N2, *Enterococcus durans* strain PL25.

Walsh *et al*., (2012) investigated the isolation of thermoduric gram-positive bacteria from several dairy products and dairy wastes like milk, cheese, and whey. Using molecular 16S rRNA gene identification, *Enterococcus durans* were recorded as one of the predominant microorganism in WPC process line.

moreover, It was reported by Shivsharan, (2014) that *Enterococcus hirae* and *Enterococcus faecium* were a dominant species which isolated from dairy effluents collected from Maharashtra, India in the period of 2011-2013.

Švec *et al*., (2006) isolated three strains in the *Enterococcus faecalis* species group. Two strains were isolated from water samples and the third was isolated from the termite sludge. By using 16S rRNA gene sequence analysis classified, the isolates identified as *Enterococcus silesiacus* sp. nov. W442T (=CCM 7319T=LMG 23085T) and *Enterococcus termitis* sp. nov. LMG 8895T (=CCM 7300T).

Moreover, Siddque and Alif, (2018) studied the isolation of some species of Enterococcus and determination their ability to Decolourization green and orange dyes textile wastewater. Out of the isolated species was *Enterococcus termitis*. This indicates the importance of Enterococcus species not only in some dairy and food production but also in the biotechnological wastewater resources.

Twenty three *Enterococcus durans* isolates collected from Moroccan dairy products were isolated by Jamaly *et al*., (2010). Strains were identified by species-specific PCR. Moreover, their technologically and biochemical characteristics were studied. The study proved that the strains displayed weak acidification and autolysis activities in milk. Although, they showed high extracellular proteolytic activity. All examined strains produced exopolysaccharides and most of them could metabolize citrate and tolerate a high concentration of nisin. The strains were not resistance to vancomycin and didn't show haemolytic activity. This leads to suggest the use of these isolates as adjunct starters in food fermentations process.

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تواجد الإنتيروكوككس تيرميتس فى شرش الجبن

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الإنتيروكوككس يعد من أكثر الأجناس البكتيريه إنتشاراً في اللبن و منتجات الألبان والمخلفات السائله اللبنيه. تهدف الدراسه الحاليه لدراسة وجود الإنتيروكوككس تيرميتس في عينات من المخلفات اللبنيه السائله كشرش الجبن والذي تم تجميعه من معامل ألبان مصريه مختلفه. تم تخطيط العينات سطحيا على أطباق من بيئة التريبتون صويا آجار بإستخدام طريقة ميلس- مسرا. 13 عزله من إجمالى 86 عزله بكتيريه من المتوقع إنتمائها لجنس الإنتيروكوككس. العزلات المتوقع كونها إنتيروكوككس تم إختبارها مورفولوجياً. بالإضافه لذلك، فالعديد من الإختبارات البيوكيميائيه تم إجرائها على العزلات مثل الكتاليز، تحلل النشا، إستخدام السترات، تحلل الكازين، ليسين دى كربوكسيليز، ليسين دى أمينيز، إنتاج كبريتيد الأيدروجين، تحلل الإسكيولين، النمو على بيئة آجار المانيتول. علاوة على ذلك فالعزلات المتوقع كونها إنتيروكوككس تم إختبار قابليتها لتخمير اللاكتوز، جلوكوز، جالاكتوز، فركتوز، دكستروز، مانوز، ريبوز، زيلوز، والمانيتول. أثبتت نتائج الإختبارات المورفولوجيه والبيوكيميائيه أن العزلات المختبره تنتمي إلى الإنتيروكوككس تيرميتس. ولمزيد من التعريف تم إجراء إختبار تفاعل البوليميراز المتسلسل كطريقه جينيه لتعريف عزلات الإنتيروكوككس تيرميتس على مستوى تحت النوع. أثبتت نتائج إختبار تفاعل البوليميريز المتسلسل وتتابع 16s RNA أن العزلات المختبره هى إنتيروكوككس تيرميتس سلالة LMG 8895 .