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MICROBIOLOGICAL QUALITY AND SAFETY OF COMMERCIAL MARKET YOGURT IN GIZA, EGYPT

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ABSTRACT

Yogurt is the most popular dairy product in Egypt. The popularity of yogurt can be attributed to its sensory characteristics and nutritional value. The microbiological characteristics of yogurt also contribute greatly to the quality and shelf life of the final product. One hundred yogurt samples were collected from local market stores and were examined for their culture viability of lactic acid bacteria (*Lactobacillus* spp. and *Streptococcus* spp.), *Bacillus* spp., psychrophilic bacteria (*Pseudomonas* spp.), pathogenic bacteria (*Salmonella* spp., *Staphylococcus* spp. and *Escherichia coli*) and some common fungi (*Aspergillus* spp. and *Penicillium* spp.) before and after chilled conditions for 10 days at 7ºC ±1. Yogurt culture population maintained a high population (7-9 log CFU/ml) after chilled storage condition with increasing the probability to prevent foodborne illness in consumers. Here, the psychrophilic bacteria ranged from 5-6 log CFU/ml, whereas the other tested groups ranged from 2-4 log CFU/ml. Storage condition period decreased the number of pathogenic bacteria which showed statistically significant level as comparing after and before storage. Our research demonstrates the importance of implementation of HACCP technique as a quality control practices to ensure the highest yogurt quality in Giza, Egypt. In addition, we report bacterial identification using MALDI-TOF MS as simple, effective, time and cost-effective technique which can be used in a different application instead of the conventional method that were cumbersome and cost-consuming.

Keywords: Yogurt, Lactic acid bacteria, pathogenic bacteria, MALDI-TOF MS

INTRODUCTION

Yogurt is one of the most popular fermented milk products known for thousands of years, especially, among the children and persons on a diet. Its fame came from its nutritional value as the constituent of basic milk products and its probiotic component (De et al., 2014). Moreover, the popularity of fermented dairy products as vehicles for the transmission of probiotics to consumers has grown (Steijns, 2008). It is also rich in multivitamins as, riboflavin, vitamin B6, and B12 as well as protein and calcium (Ashraf and Shah, 2011).

Yogurt is an excellent growth medium for a wide range of microorganisms, including fecal coliforms *E.coli*, *Salmonella* sp. and *Pseudomonas* sp., which unfortunately decrease the shelf-life of the product (Nwagu and Amadi, 2010) which lower the grade and unsafe products. Therefore, it is important to monitor and evaluate the microbiological quality and safety of yogurt by using three parameters: First: the total viable count (TVC) and the presence of the pathogenic bacteria (Erbaş et al., 2005; Senguna et al., 2009); second: the presence of lactic acid bacteria (LAB), the major components of starter cultures used in yogurt production, and. Third: the production of growth-inhibiting substances that inhibit food spoilage bacteria.

Lactic acid bacteria always occur as indigenous microflora of raw milk and as natural components of the gastrointestinal microflora (Coeuret et al., 2003). Lactic acid bacteria play an important role in the production and conservation of fermented foods, especially in the dairy industry as well as being major components of starter cultures used in fermentation. Many studies have been carried out to evaluate the microbiological quality and safety of commercial yogurt. Recently, it could be reported that total bacterial count of two type of yogurt samples were increased with microbial load of *Bacillus* sp. and *Aspergillus* sp. that present in all the samples (De et al., 2014).

The traditional phenotypic, biochemical and molecular methods of bacterial isolates are considered as time consuming and expensive methods which took from hours to weeks for positive identification. Accordingly, there is a growing need to develop a quick and accurate method for the bacterial identification, and to achieve this goal, matrix –assisted laser desorption ionization time of flight mass spectroscopy (MALDI-TOF MS) is a developing method were used for highly accurate bacterial identification however, it is safe, rapid and cost-effective (De Bruyne et al., 2011). MALDI-TOF MS could be used to obtain protein fingerprinting from whole bacterial cells, then by comparing these fingerprints to a reference database by the use of various algorithms for interpretation and comparison of these spectra (Fenselau and Demirev, 2001; Vargha et al., 2006).

Therefore, the objective of the present work is to monitor the quality and safety of market yogurt microbiologically during the refrigerated storage in Giza city, Egypt using different technique to detect bacterial contamination

MATERIALS AND METHODS

One hundred market yogurt samples were randomly collected from local market of Giza city, Egypt during the period extended from October 2013 to February 2014. Microbiological evaluation was carried out on the collected samples before storage at zero time and after 10-days of storage at 7ºC ±1.

All media, namely De Man Rogosa and Sharpe (MRS) and M17 agar media for both *Lactobacillus* and *Streptococcus* count, respectively, and selective media used for bacterial identification were purchased from Oxoid, UK. All tested sugars were purchased from Sigma, USA.:

The color, consistency of each sample was recorded after collection of samples and after storage condition at 7ºC ±1. The pH of each yogurt sample was examined using 3505 pH meter (UK). The acidity of yogurt samples after and before storage at 7ºC ±1 were measured according to (AOAC, 2000) method number 947.05 using phenolphthalein (Riediel DE HAEN AG, Germany) as an indicator.

Total bacterial count were enumerated using plate count method (Benson, 2005) before and after storage condition at 7ºC ±1. Samples were serially (10-fold) diluted in sterile potassium phosphate buffer (pH 7.0). Subsequently, 1 ml aliquots of each sample, after shaken vigorously to suspend the microbial content, were plated onto selective agar media in duplicates, followed by incubation at 37ºC for 24 h for bacterial counts and at 25ºC for 48-72 h for fungal counts. Growth was calculated as colony forming unit (CFU/ ml).

All selected isolates were microscopically examined (Olympus, Japan), and were specifically identified according to the methods described by Bergey's manual of systematic Bacteriology (Holt and Krieg, 1984; Brenner et al., 2005), while fungi were identified by the methods of Rapper and Fennell, (1965) and Thom, (1910).

All ofr the selected isolates were examined for their ability to induce fermentation of different sugars according to the methods described by (Cappuccino and Sherman, 2004).

Biochemical tests were performed to all isolates according to method described by Malik, (1995), including catalase test; oxidase test; coagulase test; indole test; urease test; Simmon's citrate utilization; methyl red test; Voges-Proskauer test and motility test. The isolates were identified by comparing their characteristics with those of known taxa, as recommended by Bergey's manual of systematic Bacteriology (Holt and Krieg, 1984; Brenner et al., 2005).

All identified bacterial isolates were confirmed using MALDI-TOF MS. Bacteria were sub-cultured and incubated overnight, then diluted to a 0.5 McFarland standard for direct colony method, bacteria were applied as a thin film onto a 96-spot steel plate (Bruker Daltonics) and allowed to dry at room temperature. Subsequently, 2 µl of MALDI matrix (a saturated solution of α-cyano-4-hydroxycinnamic acid [HCCA; Bruker Daltonics] in 50% acetonitrile and 2.5% trifluoroacetic acid) was applied onto the colony and allowed to dry. For each plate, a bacterial test standard (Bruker Daltonics) was included to calibrate the instrument and validate the run. MALDI-TOF MS was performed with the MicroFlex LT mass spectrometer (Bruker Daltonics) according to the manufacturer's suggested recommendations. Spectra were analyzed by using MALDI Biotyper automation control and the Bruker Biotyper 2.0 software and library (version 2.0, 3,740 entries; Bruker Daltonics). Identification score criteria used were those recommended by the manufacturer: a score of ≥ 2.000 indicated species-level identification, a score of 1.700 to 1.999 indicated identification to the genus level, and a score of <1.700 was interpreted as no identification.

To analyze the total count of bacterial and fungal growth after and before chilled storage at 6oC among collected samples, one-way analysis of variance (ANOVA) was conducted using IBM SPSS statistics 20. All analyzes were repeated in triplicates. The generated data were subjected to analysis of variance (ANOVA) (Armitage and Berry, 1987). Differences among mean values were established with mean values comparison using LSD's test and were considered significant at p value was < 0.05.

RESULTS AND DISCUSSION

Based on physiochemical properties, one hundred collected yogurt samples were white in color, solid, semisolid and others were watery after 10 days at 7ºC ±1 storage condition. The pH of freshly collected samples was in the range from 4.1 to 3.9 ± 0.5, and after storage, the pH slightly decreased from 4.1 to 3.6 ± 0.5. Titratable acidity of freshly collected yogurt samples ranged from 00.87 - 00.89 ± 0.02; while after storage for 10 days at 7ºC ±1 it increased from 00.90 - 00.99 ± 00.2.

The viable microbial counts of collected yogurt were carried out on different selective media for each microbial group (Table 1) at the time of purchase and after 10 days of storage at 7ºC ±1. Counts of*lactobacillus* spp. and *Streptococcus* spp. ranged from 55 X 10^6 to 86 X10^7 CFU/ ml before storage, respectively. The lactobacilli counts slightly increased from 55 X 10^6 to 96 X10^6 CFU/ml, respectively after 10-days storage period, and their counts were statistically different from their own counts before storage; whereas streptococci count slightly decreased from 86 X10^7 to 77 X10^7 CFU/ml, however, both of lactobacilli and streptococci maintained the highest value (higher than 10^6 CFU/ml), as compared to the other bacterial and fungal groups. These counts reflect the dominance of both groups in yogurt samples.

The high populations of lactic acid bacteria (lactobacilli and streptococci) in collected yogurt samples, with slightly variation that supported by recent finding (Makut et al., 2014; Yerlikaya et al., 2015) and the variations in total microbial count of collected yogurt samples could be a reflection of the manufacturing practices and preservation protocols (Nwagu and Amadi, 2010). Furthermore, the study showed after refrigerated storage, the numbers of lactobacilli and streptococci have a favorable dominance against the potential pathogenic organisms, even with the decrease in number. The present findings are in agreement with those reported with Sömer and Kılıç. (2012)

The pathogenic microorganisms (Table 1) were present in all of the collected fresh samples at a different level. Regarding the bacterial counts of Bacillus spp. and Pseudomonas spp., they were relatively moderate in the tested samples but with significant level after and before storage. As for counts of Salmonella spp., Staphylococcus spp. and E. coli, they were obvious in

collected fresh yogurt before storage, whereas a significant decrease was noticed in counts limits under the storage conditions.

The fungal groups showed lower existence, in which the counts of Aspergillus spp. are close to that obtained by pathogenic bacteria and lower than that obtained by lactic acid bacteria. On the other hand, the numbers of mold insignificantly decreased during storage at levels of 2 X 104 to 3 X 103 CFU/ml, whereas no evidence of Penicillium spp. contamination before and after storage (Table 1). Massa et al., 1997; Ng et al., 2011 demonstrated that the decrease in the microbial load may be attributed to various environmental stresses during food production and storage condition, or/and may be due to accumulation of some inhibitory metabolites such as organic acids produced during lactic acid fermentation, as recorded from pH shift, that slows down or stops the growth as recommended by Nassib et al., (2006)); Beitane and Klava, (2013), which can be also the results of decrease in pathogenic counts in yogurt samples. The antagonistic action of produced organic acid is believed to be (i) interference with the maintenance of cell membrane potential, (ii) inhibition of active transport, (iii) reduction of intracellular pH, and (iv) inhibition of various metabolites functions (Sarkar, 2007).

It could also be concluded from the data that some bacterial and fungal pathogens could tolerate the acidic conditions created by starter cultures (Gahan et al., 1996; Hal-Haddad, 2003) thus cause survival in yogurt, or the use of contaminated raw material as flavors, and colorings besides equipment used in storage of the milk and its handling (Bramley and Mckinnon, 1990; Beitane and Klava, 2013; El-Malt et al., 2013). In addition, temperature and duration of storage of the milk can cause its contamination (Boor and Murphy, 2002; Huck et al., 2008) also inadequate post fermentation hygiene prior to packaging or their ability to adapt differential conditions such as transportation process and/or packaging, storage, transport and sales (De et al., 2014).

Accordingly, fermented products manufactured with starter cultures should not be assumed to be free of pathogen. In this regards, the incidence of some pathogenic bacteria under cold storage condition was observed, similarly with other reports (Aguoru and Dania, 2010; Issazadeh et al., 2012). However, we suggested that to control microbial contaminations in food, it is essential to control the hygiene and sanitation procedures during manufacturing with the selection of good quality of raw materials (Mayoral et al., 2005).

Table (2) shows the obtained data, depending on different traditional methods for identification. Out of 100 yogurt samples 72 bacterial isolates were isolated, 25 bacterial isolates were belonging to *Lactobacillus* sp. which identified as: *L. bulgaricas*, *L. delbrueckii***,** *L. acidophilus*, *L. plantarum* and *L. casei*. In addition, 18 bacterial isolates were belonging to *Streptococcus thermophiles* according to Bergey's Manual of Determinative Bacteriology; while for pathogenic bacteria ten *E.coli* isolates, eight *Pseudomonas* spp., five *Bacillus* spp., four *Staphylococcus aureus* and two *Salmonella* sp. were isolated. Also fungal identification, seven *Aspergillus* spp. were identified.

In order to compare the bacterial identification using phenotypic traditional methods with MALDI-TOF MS, the obtained data in Table (3) revealed that the Bruker Biotyper exhibited better performance for identifying some isolates to the genus and species level (97.22% and 88.88%, respectively); while the incorrect identification was (2.77% and 11.11%) for genus and species, respectively. Additionally, Bruker Biotyper system achieved sixty (60) isolates that were identified with high confidence to the species level using single direct colony analysis. Twelve (12) isolates yielded wrong identification in species level despite have a score of ≥ 2.000 and with reanalysis the 12 isolates by second direct colony analysis revealed that four isolates were correctly identified to species identification and the other eight isolates had no improvement in identification. For genus level, nine isolates having an initial score of < 2.000 were then reanalyzed. Concerning second direct colony analysis, only 7 isolates had a score of ≥ 2.000 and two were misidentified (score, < 1.700) as represented in Table (3). For *Aspergillus* sp. identification using MALDI-TOF MS, no results could be achieved.

Table (1). Type and count (CFU/ml) of microbial groups found in collected yogurt before and after storage under chilled condition

Type of microorganisms	Total microbial counts (CFU ml^{-1})[1]	
	Before storage	After storage
Lactobacillus spp.	55 X 10^6±2.88^a	96 X10^6 ±0.0^b
Streptococcus spp.	86 X10^7±6.66^c	77 X10^7±1.45^d
Bacillus spp.	21 X10^4±5.77^e	59 X10^6±5.77^b
Pseudomonas spp.	12 X 10^5±1.15^e	86 X10^6±1.0^d
Salmonella spp.	2 X 10^6±0.0^e	6 X10^3±1.15^f
Staphylococcus aureus	3 X 10^4±0.0^e	7 X10^2±5.77^f
E. coli	5 X10^3±5.77^e	3 X10^3±0.0^f
Aspergillus spp.	2X10^4±0.0^e	3 X10^3±5.77^f
Penicillium spp.	00.0	00.0

[1] **Means with the different letter within the same column were significantly different (p < 0.5)**

Table (2). Biochemical test and sugar fermentation of isolated microorganisms

Characteristics	*L. bulgaricas*	*L. delbrueckii*	*L. acidophilus*	*L. plantarum*	*L. casei*	*Streptococcus spp.*	*E.coli*	*Salmonella spp.*	*Staphylococcus aureus*	*Pseudomonas spp.*	*Bacillus spp.*
Biochemical tests											
Catalase test	-[2]	-	-	-	-	-	-	-	+	+	+[3]
Oxidase test	-	-	-	-	-	-	-	-	-	+	+
Motility	-	-	-	-	-	-	+	+	-	+	+
Acid, from glucose	+	+	+	+	+	+	+	+	+	+	-/+
Gas from glucose	-	-	-	-	-	+	+	+	-	-	-
Indol test	-	-	-	-	-	-	+	-	-	-	-
Vogues prosquer	-	-	-	-	-	+	-	-	+	-	+
Methyl red	+	+	+	+	+	-	+	+	+	-	-
Urease test	-	-	-	-	-	-	-	-	-	-	-
Simmon's citrate	+	+	+	+	+	+	-	+	ND[4]	+	+
Sugar fermentation											
Glucose	-	-	+	+	+	+	+	+	+	+	+
Fructose	-	-	-	+	+	+	+	+	+	-	+
Galactose	-	-	+	+	+	+		+	+	-	+
Maltose	+	-	-	+	+	+	+	+	+	-	+
Lactose	+	-	+	+	+	+	+	-	+	-	+
Sorbitol	-	-	-	+	+	-	-	+	-	-	+
Xylose	-	-	-	+	+	+	+	+	ND	-	+
Rhamnose	-	-	+	+	+	ND	-	+	-	-	ND
Manitol	-	-	-	+	+	-	+	+	+	-	+

Table (3). MALDI-TOF MS identification of bacteria by Bruker Biotyper:

Microrganism name	No. of isolates	No. of isolates identified by Bruker Biotyper	
		Genus	Species
L. bulgaricas	2	2	0
L. delbrueckii	4	2	0
L. acidophilus	2	2	2
L. plantarum	10	10	10
L. casei	7	7	7
Streptococcus spp.	18	18	17
E.coli	10	10	10
Salmonella spp.	2	2	2
Staphylococcus aureus	4	4	4
Pseudomonas spp.	8	8	8
Bacillus spp.	5	5	4
Total no. of isolates (% correct identification)	72	70 (97.22%)	64 (88.88%)

[2] **(+) = positive result**
[3] **(-) = negative result**
[4] **ND = not determined**

In this study, as comparing between old and new method for identification, it could be observed that MALDI-TOF MS could be used as replacement or compliment for phenotypic and molecular methods for bacterial identification (De Bruyne et al., 2011; Guo et al. 2014; Bizzini et al., 2010; Bizzini and Greub, 2010). By using single direct colony analysis, 60 of 72 isolates (score, ≥ 2.000) have been identified within 1h. However, further extraction of isolates generated only a species level in 8 cases, this data suggested that the extraction did not improve the overall level identification. The isolates that were misidentified may be from generated score very close to another but give different species or genus that result from the heterotypic synonyms or absence of spectra within data (Bizzini and Greub, 2010). In addition, some studies reported that culture condition of isolates could affect the result of misidentification of MALDI-TOF MS (Bizzini et al., 2010).

CONCLUSION

It could be concluded that high count of lactic acid bacteria in yogurt samples improve health benefits of yogurt under cold storage by affecting pathogenic survival. Additionally, this high-throughput assessment explores the importance of having standardized hygienic quality control practices to ensure the highest market yogurt quality and maintain its health benefits. Moreover, MALDI-TOF MS is an effective method for bacterial identification due to its simplicity, effective, time and cost-effective technique and can be used in different application.

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الجودة الميكروبية والأمان لمنتجات الزبادى التجارية فى مدينة الجيزة – مصر

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يعتبر الزبادي من أشهر منتجات الالبان في مصر و تعود شهرته الى قيمته الغذائيه ومذاقه المميز. كما أن خصائصه الميكروبيولوجيه تعود إلى جودته و تاريخ صلاحيته كمنتج نهائي. في هذه الدراسه تم تجميع مائه عينه من الزبادي و تم دراسه المحتوى الميكروبي من السلالات الميكروبيه المختلفه مثل خميره البكتريا (اللاكتوبسلس, الاستربتوكوكس), جنس الباسلس, الساكروفيليك بكتريا (جنس السودوموناس, البكتريا الممرضه (السلمونيلا, ستافيلوكوكس, الاشريشيا كولاي) و بعض انواع من الفطريات (الاسبرجيلس, البنيسيليم) قبل و بعد التخزين في الثلاجه عند درجه حراره ١±7 C° لمده ١٠ أيام التي ساهمت في الحفاظ على معدل تعداد خميره البكتريا في الزبادي مع تجنب احتماليه تلوث المنتج. الدراسه أوضحت أن تعداد الساكروفيليك بكتريا يتراوح ما بين ٥-٦ لوغاريتم خليه بكتيريه / مل بينما تعداد الانواع البكتيريه الاخرى ما بين ٢-٤ لوغاريتم خليه بكتيريه / مل. أظهرت الاحصائيات أن التخزين عند درجات الحراره المنخفضه كان لها تأثير في تقليل تعداد البكتريا الممرضه في الزبادي لوجود زيادة فى نمو بكتيريا حامض اللاكتيك من جنس اللكتوباسيلس. أظهرت الدراسه أيضاً بضرورة تطبيق نظام تحليل المخاطر و نقاط التحكم الحرجه (HACCP)لضمان نقاوة المنتج . كما تم في هذه الدراسه أيضا إستخدام تقنيه الملدي توف ماس سبكتروسكوبي الحديثه لتعريف البكتريا و التي تتميز بأنها بسيطه, سهله, سريعه و غير مكلفه مقارنه بالطرق التقليديه القديمه التي كانت مرهقه و مكلفه والتى كانت تأخذ وقت طويل فى التعرف على الكائنات الحية الدقيقة.