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RESEARCH ARTICLE OPEN ACCESS

Molecular Characterization and Biofilm Formation Study of Contaminant Bacteria Isolated from Domiaty and Hungarian Cheeses in Jeddah City

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Abstract

The aim was to study the microbiological quality of Domiaty and Hungarian cheeses, molecular identification and biofilm formation of some selected contaminant bacteria. Samples were collected from two M and P big markets in Jeddah City through the period from February to October 2018, nine visits for two types of natural cheese. Results showed that the total bacterial counts (CFU/ml) from Domiaty cheese from two markets (M and P) were 0.1 x 10^5, 8 x 10^5 and 1 x 10^5 CFU/ml respectively (3 visits of M market) and 4 x 10^6, 0.4 x 10^6, 6.5 x 10^3, 1 x 10^3, 0.1 x 10^3 and 0.1 x 10^3 CFU/ml respectively (six samples from 6 visits from P market). Results showed that the total bacterial counts (CFU/ml) from Hungarian cheese were 1.5 x 10^5, 1x 10^4, 11 x 10^4 and 4 x10^6 CFU/ml respectively from (4 visits of M market) and 0.18 x 10^4, 3 x 10^6, 22 x 10^6, 6 x 10^6 and 5 x 10^4 CFU/ml respectively (5 visits from P market).Different bacterial isolates from cheese were identified by morphology and biochemical test. Bacterial isolates from cheeses were identified by VITEK MS as follow: *Serratia liquefaciens* (D6-1, D6-2, D14-1, D13-1 and D13-2), and *Pseudomonas fluorescens* (D14-2) were isolated from Domiaty cheese while *Enterococcus faecium* (H11-2), *Serratia liquefaciens* (H15-1) and *Streptococcus thermophilus* (H14-1) were isolated from Hungarian cheese. Some selected bacterial isolates were identified by 16S rRNA. Isolates were belong to MK757978 (*Raoultilla terrigena* (D15-1)), MK757979 (*Bacillus cereus* (D16-1)), MK757980 (*Enterococcus faecalis* (H10-2)), MK757982 (*Enterococcus fiscalism* (H11-1)), MK757981 (*Serratia liquefactions* (H13-1)), MK757984 (*Anoxybacillus flavithermus* (H17-1). All bacterial isolates have been tested for the formation of biofilm using a Tissue Culture Plate (TCP). Results revealed 12.5% and 46.15% of high biofilm formation respectively for bacterial isolates of Domiaty and Hungarian cheeses.

Keywords: Domiaty and Hungarian cheeses, *S. liquefaciens, P. fluorescens, Anoxybacillus flavithermus,* 16S rRNA, biofilm detection

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INTRODUCTION

The food is the fuel of our life and it is a major concern for quality and safety[1]. Cheese is most common in Saudi Arabia, because of its health benefits and flavor, also it is a rich source of dietary calcium, proteins, and phosphorus[2]. The microbial contamination in the cheese may arise from different sources, these sources during the cheese production as: ground, starter culture, brine, packaging materials, cheese cloth, yogurt cut knife, cold room and air room production (Temelli *et al*). There are several factors responsible for Domiaty cheese microbiological quality such as the thermal treatment of the milk, the raw milk, and the level and type(s) of microbial contamination that occur throughout the manufacture and cheese storage as reported by Bintsis and Papademas[3]. Domiaty cheese is one of the most popular varieties of cheese, if contaminated, it causes of foodborne illness. Cokal et al[4] reported that *(Staphylococcus aureus, Escherichia coli O157:H7, Salmonella spp.* and *Listeria monocytogenes)* were foodborne pathogens that the most common and responsible to outbreaks associated with cheese. According to[5], the cheese should be free from pathogens such as, *Staphylococcus aureus, Salmonella sp, Clostridium botulinum, Listeria monocytogenes, Campylobacter jejuni, Bacillus cereus, Escherichia coli O157:H7, Sreptococcus faecalis* and indicator hygiene include Coliform group and fungi shouldn't exceed 10 cfu/g and the yeast shroud not exceed 400 cfu/g. according to the manufacturing processes, there are many subtypes of Domiaty cheese.

Different factors, control growth pathogens on cheese include organic factor, PH value, moisture, salt concentration, temperature and hygienic control on the diary plant[6, 7]. Cheese consider as a good bacterial growth medium due to the content of nutrients and long storage duration, and several steps in production may cause bacterial risks[8]. Cheese contamination can occur with foodborne pathogens in several stages in cheese processing, as pastoralized milk, row milk, after pastoralized milk[9]. Foodborne pathogens contaminated different types of cheeses as *Staphylococcus aureus*, *Listeria monocytogenes, Salmonella* spp. and *E. coli*. *S. aureus, Salmonella* spp*. or E. coli* can be transferred by Food-borne outbreaks occur from eating food contaminated with these pathogens that lead to serious illness[10]. Several lactic acid bacterial species from Domiaty cheese were isolated and identified, such as *Lactobacillus delbrueckii* subsp. *Bulgaricus, Lactococcus lactis* subsp. *lactis*, *L. casei* as reported by Fahmy and Youssef [11] and *Enterococcus faecalis*, *E. faecium* and *L. farciminis*, *L. alimentarius* as reported by El-Zayat et al[12] and EL-Hamshary et al[13] isolated different bacterial strains from white cheese *B. cereus* (S1) *Staphylococcus aureus* (S2); *Bacillus paramycoides* (S3); *Staphylococcus aureus* (S5); *Serratia proteamaculan* (S6); *Serratia proteamaculan* (S7) and *Serratia proteamaculan* (S9)).

A biofilm consists of one or more of bacterial strains in extracellular polymeric substance (DNA, protein or carbohydrates) matrix[14], or as reported by Satpathy *et al.* that bacterial strains bind to surfaces and form spatially structured communities inside a self-produced matrix, containing extracellular polymeric substances (EPS) known as biofilms. Also, Wingender and Flemming[15] reported that extracellular polymeric substances (EPS) are biosynthetic polymers produced by micro-organisms from prokaryotic, and the production of EPS by bacterial strains in (culture or aggregates) is affected by the microbial species, phases of growth, nutritional status and the conditions of environment[16]. Bacterial EPS affect cell adhesion, microbial aggregates formation (biofilms, flocs, sludges and bio-granules), as reported by Comte et al[17]). Biofilms are very important for the industry of food because biofilms make bacteria to bind to a number of surfaces, including food products, rubber, polypropylene, plastic, glass, stainless steel, and through just a few minutes, then is followed by mature biofilms developing (a few days or hours)[18], Food processing lines are a suitable environment for biofilms to form on food contact surfaces, primarily due to manufacturing plants' complexity, long production periods, mass product generation, and large biofilm growth areas[19]. Many food-borne bacteria may, therefore, bind to the contact surfaces present in these areas, which could contribute to increase the risk of bacterial food-borne diseases. 80% of bacterial infections for example in the USA are believed to

be related specifically to food-borne pathogens in biofilms[20].

In the industry of food, species that forming biofilm appear in environments of factory and can be pathogenic to humans because they develop biofilm structures. The processing environments of the food industry, e.g., wood, glass, stainless steel, polyethylene, rubber, polypropylene, etc., act as artificial substrates for these pathogens as reported by Abdallah et al[21] and Colagiorgi[22].

The characteristics of attachment surface's affect the production of mixed-species biofilm[23], conditions of environment[24], and involved bacterial cells[25,26]. Food matrix components[27], in food processing environments also influence attachment of bacteria[28]; e.g., food waste, such as exudates of milk and meat enriched in fats, carbohydrates and proteins, facilitate microorganism multiplication and growth, and favors dual-species biofilm development by *E. coli* and *Staphylococcus aureus*[29,30] reported that milk lactose improves biofilm production by *Bacillus subtilis,* by activating the LuxS-mediated quorum-sensing system, and *S. aureus* through development intercellular polysaccharide adhesion[31].

Lafarge et al[32] detected *Serratia* spp. bacterial strains in different sources as raw milk, in a milk-processing plant[33], milk bulk tank as reported by Decimo[34], and from internal surfaces of tankers of raw milk and reported that produce (heat-resistant proteolytic enzymes) and it is included in monitoring the refrigerated raw milk quality, and biofilms producer in single culture and in mixed with *Streptococcus uberis* on the stainless-steel surfaces[35,36], and *Serratia spp*. possess forming biofilm much higher than for *Pseudomonas spp*. and showed that Serratia isolates were found as one of the most predominant proteolytic enzymes producers *Pseudomonas* spp. biofilms tended to have a smaller ratio of mass: cells and mixed with *Serratia* spp., presenting the opposite pattern as reported by Cleto et al[33]. The presence of a single different strain may have a significant effect on the microbial dynamics in dairy products[32].

Machado et al[37] reported that in dairy products, the dynamics of a microbial population have been studied by molecular methods, based on sequencing a fragment of 16S rDNA gene and comparing with NCBI databases. The most proteolytic isolates were selected for identification using 16S rDNA sequencing. *Serratia liquefaciens* (73.9%) and *Pseudomonas spp.* (26.1%) were identified as the dominant psychrotrophic microorganisms with high spoilage potential. The milk spoilage microbiota knowledge will be important for improve milk and dairy products quality. *Serratia liquefaciens* is a spoilage microorganism of relevance in the dairy industry because it is psychrotrophic, biofilm producer, and produces thermoresistant lipases and proteases[38], and from milk as showed by Gaffer et al[39].

Bacillus cereus is a Gram-positive and spore-forming bacterium that can grow in various environments at wide-ranging temperatures (4°C-50°C), and It is resistant to (chemicals, radiation and heat treatment)[40]. Pathogenic bacteria as *Bacillus cereus* was detected in three samples of cheese[41], *Bacillus cereus*is a frequently isolated from food and food products, dairy products, it secretes toxins that can cause sickness and diarrhoea symptoms in humans. *B. cereus* is responsible for biofilm formation on food contact surfaces, such as stainless steel pipes, conveyor belts and storage tanks. It can also form floating or immersed biofilms, which can secrete a vast array of bacteriocins, metabolites, surfactants, proteases and lipases, in biofilms, which can affect qualities of food[42]. Motility by bacterial flagella confers access to suitable biofilm formation surfaces, and is required for biofilms to spread on non-colonised surfaces. However, *B. cereus* flagella have not been found to be directly involved in adhesion to glass surfaces, but can play a key role in biofilm formation via their motility[43]. *B. cereus* that contaminates both milk and milk products is based on the fact that usually contaminate milk during milking or storage on the farm, then gain entrance to dairy products from which they are prepared that depends on the effectiveness of hygienic measures applied during, handling, processing and distribution products of milk[44].

Oliveira et al[45] evaluated multispecies biofilms formed on stainless steel (SS) due to the contaminating microbiota in raw milk and genetic diversity analysis indicated that Gammaproteo bacteria and Bacilli predominated in the biofilms, they have spoilage potential and

they representatives of great importance. The biofilms can be formed on the surfaces of dairy processing equipment and are a potential source of product contamination. *Pseudomonas* spp. produce EPS huge amounts and are known to attach stainless steel surface and form biofilms. They can co-exist in biofilms with other pathogens to form multispecies biofilms, which make them more resistant and stable[46]. These biofilms can be accompanied by a distinct blue discolouration (pyocyanin) on fresh cheese produced by *P. fluorescens*[47].

Anoxybacillus flavithermus is Gram-positive, thermophilic, and spore-forming organism that is facultatively anaerobic and non-pathogenic[48]. *A. flavithermus* spores are resistant to heat and their vegetative cells can grow at temperatures up to 65°C with a significant increase in bacterial adhesion on stainless steel surfaces in the presence of skimmed milk, and this indicator that milk positively influences these species' biofilm formation[49]. The commonest isolates that producing biofilm are thermophilic genera in the dairy industry as reported by Burgess et al[50]. It is essential that Biofilm-related effects in food industries as (pathogenicity, corrosion of metal surfaces, and alteration to organoleptic properties based on proteases or lipases secretion) are critically important. For example, in the dairy industry several structures and processes (pipelines, raw milk tanks, butter centrifuges, pasteurisers, packing tools, cheese tanks) can act as biofilm production surface substrates at different temperatures and involve several mixed cell species. Thus, to avoid contamination and to ensure food safety in the food industry, accurate methods to visualise biofilms in situ be set up[51]. For fighting biofilms[52] reported that two strategies in the industry of food: structural modification of surfaces or application of antibacterial or antibiofilm coatings[53]. Thus, several alternative products to classic disinfectants (chlorine, quaternary ammonium, etc.), such as, plant-derived antimicrobials being the compounds that display more significant antimicrobial action in shorter action times as reported by EL-Hamshary et al[13] that ethanolic and ethyl acetate extracts of Tamarix nilotica plant showed antibacterial activity against *B. cereus* (S1) *Staphylococcus aureus* (S2); *Bacillus paramycoides* (S3); *Staphylococcus aureus* (S5); *Serratia proteamaculan* (S6); *Serratia proteamaculan* (S7) and *Serratia proteamaculan* (S9)) bacterial strains.

The aim of work is to study the prevalence of bacterial contamination in Domiaty and Hungarian cheeses collected from two big markets in Jeddah City. Identification of bacterial isolates by morphological characterization, biochemical test, biomerieux Vitek MS and molecular identification by 16S rRNA gene. The ability of bacterial isolates (29 bacterial isolates were tested for produce biofilm (16 Domiaty and 13 Hangarian cheese) using Tissue Culture Plate (TCP) quantitative technique.

MATERIAL AND METHODS

Media preparation

Different media were used as nutrient agar (NA)[54], MacConkey agar adjusting pH to 7.4[54]. All media during the present study were sterilized by autoclaving at 121°C for 2hrs and used for bacterial growth experiments.

Collection of cheese samples

Domiaty and Hungarian cheeses were collected from two markets (M and P) in Jeddah city. Samples transported aseptically in ice container under refrigeration temperature 4°C to be tested immediately at the laboratory.

Isolation of bacterial isolates

Preparation of samples

One gram from each cheese sample was taken from the upper surface and blended with 9 ml of sterile distilled water in falcon tube were prepared on serial dilution method from 10^{-1} until 10^{-7} and 100 microliter of each dilutions were spread on top of the nutrient agar (NA) medium then incubated at 37°C for 24 to 48 hrs.

Viable bacterial counts

This method was used to enumerate the total count of viable bacteria, bacterial colony were picked up after 24 to 48 hrs. on (NA) from each diluted cheese sample. Colonies were counted (total cell count) and the results were expressed as (C.F.U/ml) estimated on standard plate count (SPC)[55].

Bacterial isolation and purification

Specific bacterial colonies were selected according to morphological study such as: color,

size and margin, then isolated and purified by repeated streaking on the (NA) agar medium plate and incubated at 37°C for 24 to 48 hrs to obtain pure single colony.

Morphological characterization of bacterial isolates

Gram staining of isolates of bacterial was carried out using method as reported by Allan et al[56].

Biochemical identification

Indol test

Indole test determines the ability to decomposing microorganism amino acid tryptophan to indole. Bacterial isolates from cheeses were grown on NB medium for 24 to 48 hrs. at 37°C before used. Indole urease medium of indole test was prepared and 5 ml was fill to all test tubes then transfer one ml from each bacterial isolate test tube, and uninoculated tube was kept as control. If tryptophan oxidized by bacteria, cherry red color was appeared on the top layer that indicated a positive result while if cherry red color wasn't appeared that indicated negative result[57].

Catalase test

Catalase test facilitates to detect the presence of catalase enzyme. This enzyme produced by bacteria which use oxygen in respiration. Catalase enzyme break down hydrogen peroxide H_2O_2 into water and hydrogen. Single colony from fresh bacterial isolates that grown on NA and transferred on clean glass slide then a drop of 30% [v/v] H_2O_2 solution was placed on it. Appearance of bubbles indicated positive result (CAT+) while no bubbles mean negative result (CAT-)[58].

Oxidase test

This test used to determination the presence of cytochrome enzyme oxidase in bacteria. The reagent used is a dye (TMPD) acts as an artificial electron accepter substituting the oxidase. Single colony from fresh bacterial isolates that grown on NA medium. Cotton swaps dipped in oxidase reagent (TMPD) then touched the colony of fresh selected isolates to test them. Blue-purple color appeared on filter paper mean oxidase positive, while yellow color mean oxidase negative[59].

Starch hydrolysis

This test examined the ability of isolate to produce α-amylase on medium containing starch as carbon source. The bacterial isolates were grown on starch nitrate agar medium at 37°C for 2 days. All plates were flooded with iodine solution for 3 minute appearance of clear zone around the growth indicated the starch hydrolysis while blue color mean no hydrolysis[60].

Identification of bacterial isolates

Identification by biomerieux VITEK® MS compact system

VITEK MS is an automated mass spectrometry microbial identification system that uses Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) technology (MALDI-TOF) has been shown to be both accurate in the identification of bacteria and rapid[61]. The methods as described by Westblade et al[62].

Molecular identification of isolats based on 16S rRNA sequencing

Bacterial colonies isolated from cheese samples were molecularly identified using sequencing of the 16S rRNA. GeneJET Genomic DNA extraction kit used for extract genomic DNA according to the manufacturer's instructions. DNA extracted were amplified by polymerase chain reaction (PCR) using 16S rRNA universal primer pair (The forward primer 27F 5' (AGA GTT TGA TCM TGG CTC AG) 3 and reverse primer 1492R 5'(TAC GGY TAC CTT GTT ACG ACT T)3') to amplify the 16s rRNA gene. 29 bacterial isolates were tested for produce biofilm formation (16 Domiaty and13 Hangarian cheese) using Tissue Culture Plate (TCP) quantitative technique then sequences compared with the available sequences against the 16S rRNA sequences database using NCBI's Blast N.(www.ncbi.nlm.nih.go).

Biofilm detection method

Tissue culture plate (TCP)

Biofilm assay was performed based on growth and biofilm formation of bacteria in 96 well microtiter, Tissue culture plate (TCP) is considered as a standard test for the detection the production of biofilm. The overnight cultures grown in NB were diluted at 10^{-3} and inoculated into six individual wells of a Tissue Culture Plate Method (150µl per well). Then the plates were incubated for 24 hrs. at 30 C. The ability of bacterial isolates (29 bacterial isolates were tested for produce biofilm (16 from Domiaty and 13 from Hangarian cheese) using Tissue Culture Plate (TCP)

quantitative technique as described by Mathur et al[63].

RESULTS AND DISCUSSION

Collction of cheese sampels

Eighteen samples of Domiaty and Hungarian cheeses were obtained from two big markets (M and P) in Jeddah City at a period between February to October 2018.

Isolation of bacterial isolates from cheese samples

Results in Table (1) showed the total bacterial counts (CFU/ml) from Domiaty cheese from two markets (M and P). The results indicated that the number of bacterial isolates were 0.1 x 10^5, 8 x 10^5 and 1 x 10^5 CFU/ml respectively from 3 visits of M market. Six samples from 6 visits were collected from P market. The results revealed that the number of bacterial isolates were 4 x 10^6, 0.4 x 10^6, 6.5 x 10^3, 1 x 10^3, 0.1 x 10^3 and 0.1 x 10^3 CFU/ml respectively. Results in Table (2) showed the total bacterial counts (CFU/ml) from Hungarian cheese from M and P markets. The results indicated that the number of bacterial isolates were 1.5 x 10^5, 1x 10^4, 11 x 10^4 and 4 x10^6 CFU/ml respectively from 4 visits of M market. The results revealed also that the number of bacterial isolates were 0.18 x 10^4, 3 x 10^6, 22 x 10^6, 6 x 10^6 and 5 x 10^4 CFU/ml respectively from 5 visits were obtained from P market.

Minimum (Min) bacterial count of Domiaty cheese from M market was 0.1 x 10^4 CFU/ml, and Maximum (Max) was 8 x 10^4 CFU/ml. (Min) bacterial count of Domiaty cheese from P market

Table 1. Total bacterial count (CFU/ml) of Domiaty cheese

Number of visits	CFU/ml
V1 (M)	0.1 x 10^5
V2 (M)	8 x 10^5
V3 (M)	1 x 10^5
V4 (P)	4 x10^6
V5 (P)	0.4 x10^6
V6 (P)	6.5 x 10^3
V7 (P)	1 x10^3
V8 (P)	0.1 x10^3
V9 (P)	0.1 x10^3

Table 2. Total bacterial count (CFU/ml) of Hungarian cheese

Number of visits	CFU/ml
V1 (M)	1.5 x 10^5
V2 (M)	1x 10^4
V3 (M)	11 x 10^4
V4 (M)	4 x10^6
V5 (P)	0.18 x10^4
V6 (P)	3 x 10^6
V7 (P)	22 x 10^6
V8 (P)	6 x 10^6
V9 (P)	5 x 10^4

Table 3. Morphological characterization of bacterial isolates from Domiaty cheese

Number of isolates	Cell shape	Gram stain	Mackonckyagar	Morphological characterization			
				Shape	Margin	Color	size
D6-1	Bacilli	-	+	Circular	Entire	Cream	Big
D6-2	Bacilli	-	+	Circular	Entire	Cream	Small
D11-1	Bacilli	+	-	Circular	Entire	Cream	Big
D11-2	Bacilli	+	-	Circular	Entire	Cream	Small
D12-1	Bacilli	+	-	Circular	Entire	White	Small
D12-2	Bacilli	+	-	Circular	Entire	White	Small
D13-1	Bacilli	-	+	Circular	Entire	Cream	Small
D13-2	Bacilli	-	+	Circular	Entire	Cream	Small
D14-1	Bacilli	-	+	Circular	Entire	Cream	Big
D14-2	Bacilli	-	+	Circular	Entire	White	Medium
D15-1	Bacilli	-	+	Circular	Entire	Cream	Big
D15-2	Bacilli	-	+	Circular	Entire	White	Big
D16-1	Bacilli	+	-	Irregular	Entire	White	Big
D17-1	Bacilli	+	-	Irregular	Entire	White	Big

was 0.1 x10^2 CFU/ml and (Max) was 8 x 10^4 CFU/ml. This result is lower than the similar studies although[64], collected Domiaty cheese from Cairo and Giza, results indicated the total bacterial count per gram CFU/g. At Cairo, (Min) bacterial count of Domiaty cheese was 9x10^2 CFU/g and (Max) bacterial count was 3x10^6. From Giza Minimum (Min) bacterial count of Domiaty cheese was 7x10^2 CFU/g and Maximum (Max) bacterial count was 2x10^8. Hungarian cheese obtained from M market and results indicated that CFU/ml were 1.5 x 10^4, 1x 10^3, 11 x 10^3 and 4 x10^5 obtained respectively while from P market, samples of Hungarian cheese obtained and results indicated that CFU/ml were 0.18 x10^3, 3 x10^5, 22 x10^5, 1 x10^2, 6 x10^5 and 5 x10^5 respectively. (Minimum) bacterial count of Hungarian cheese from M market was 1 x10^3 CFU/ml and (Maximum) was 4 x 10^5 CFU/ml. whereas from P market (Min) was 1 x10^2 CFU/ml and (Max) was 22 x10^5 CFU/ml. These results are similar and higher than in bacterial count to that reported by Alper and Nesrin[65], that indicated the total bacterial count of cheeses isolated from Turkey were 5.2 x10 ^{4} and 5.68 x 10^{11} CFU/g.

Morphological Characterization of bacterial isolates

Morphological characteristics of isolates from Domiaty and Hungarian cheeses were summarized and presented in Tables (3 and 4) respectively. Results in Table (3) shows morphological characteristics of bacterial isolates obtained from Domiaty cheese. Results showed that 6 of bacterial isolates Gram-positive bacilli and 8 bacterial isolates Gram-negative bacilli. Result in Table (4) shows morphological characteristics of bacterial isolates obtained from Hungarian cheese. Results revealed that (three of bacterial isolates Gram-positive bacilli, 1 of bacterial isolates Gram-negative bacilli and 6 of bacterial isolates Gram-positive coccus). These isolates represented 38.46%, 7.69% and 53.84% respectively.

Biochemical test of bacterial isolates from cheeses

Sixteen bacterial isolates of Domiaty cheese and thirteen bacterial isolates of Hungary cheese were tested for indole, catalase, oxidase, gelatin liquefaction and starch hydrolysis. Results of biochemical test of bacterial isolates from Domiaty and Hungarian cheese showed at Table (5).

Identification bacterial isolates

Identification by *biomeriex* Vitek MS compact system

Results of identification bacteria isolates by *biomeriex* Vitek MS *compact system* were shown in (Table 6). Six of bacterial isolates from Domiaty cheese were identified as (5 *Serratia liquefaciens*(D6-1, D6-2, D14-1, D13-1 and D13-2) and one *Pseudomonas fluorescens*(D14-2)) strains. Results showed that 3 isolates of bacteria were identified as (one *Enterococcus faecium* (H11-2), one *Serratia liquefaciens* (H15-1) and one *Streptococcus salivarius* spp. Thermophilus (H14-1)) strains isolated from Hungarian cheese.

Table 4. Morphological characterization of bacterial isolates from Hungarian cheese

Number of isolates	Cell shape	Gram stain	Mackonckyagar	Morphological characterization			
				Shape	Margin	Color	size
H6-1	Coccus	+	-	Circular	Entire	White	Small
H6-2	Coccus	+	-	Circular	Entire	Cream	Big
H10-1	Coccus	+	-	Circular	Entire	Cream	Small
H10-2	Coccus	+	-	Circular	Entire	Cream	Medium
H11-1	Coccus	+	-	Circular	Entire	White	Small
H11-2	Coccus	+	-	Circular	Entire	White	Medium
H13-1	Bacilli	-	+	Circular	Entire	White	Small
H14-1	Strepto- coccus	+	-	Circular	Entire	Cream	Small
H15-1	Bacilli	-	+	Circular	Entire	White	Small
H16-1	Bacilli	+	-	Circular	Entire	White	Small
H17-1	Bacilli	+	-	Circular	Entire	White	Small
H17-2	Bacilli	+	-	Circular	Entire	White	Small

Molecular identification of isolates based on 16S rRNA gene

Sequence analysis of the 16S rRNA gene has been measured fast and precise technique to recognize the phylogenetic position of bacteria. Then sequences were submitted to GenBank at NCBI web site (www.ncbi.nlm.nih.gov) under accession numbers: MK757978 (*Raoultilla terrigena*(D15-1)), MK757979 (*Bacillus cereus* (D16-1)), MK757980 (*Enterococcus faecalis* (H10-2)), MK757982 (*Enterococcus fiscalism* (H11-1)), MK757981 (*Serratia liquefactions*(H13-1)), MK757984 (*Anoxybacillus flavithermus* (H17-1)). Results of Blast search for DNA sequence in NCBI Genbank were shown in Table (7).

Biofilm detection method

In food industries, the effects related biofilm as corrosion of metal surfaces,

Table 5. Biochemical test of bacterial isolates from Domiaty and Hungarian cheese

Number of isolates	Indole test	Catalase test	Oxidase test	Starch hydrolysis	Gelatin hydrolysis
D6-1	-	+	+	-	+
D6-2	-	+	+	-	+
D10-1	-	-	-	-	-
D10-2	-	-	-	-	-
D11-1	-	+	-	-	-
D11-2	-	+	-	-	-
D12-1	-	+	-	-	-
D12-2	-	+	-	-	-
D13-1	-	+	+	-	+
D13-2	-	+	+	-	+
D14-1	-	+	+	-	+
D14-2	-	+	+	-	-
D15-1	-	+	-	-	-
D15-2	-	-	+	-	-
D16-1	-	+	-	+	-
D17-1	-	+	-	+	-
H4-1	-	-	-	-	-
H6-1	-	+	-	-	-
H6-2	-	+	-	-	-
H10-1	-	-	-	-	-
H10-2	-	-	-	-	-
H11-1	-	-	-	-	-
H11-2	-	-	-	-	-
H13-1	-	+	+	-	+
H14-1	-	-	-	-	-
H15-1	-	+	+	-	+
H16-1	-	+	-	-	-
H17-1	-	+	-	-	-
H17-2	-	+	-	-	-

Table 6. Identification bacterial genus/species isolated from Domiaty and Hungary cheeses by Vitec MS

Types of cheese	Bacterial Genus/Species
Domiaty cheese	*Serratia liquefaciens* (D6-1, D6-2, D14-1, D13-1 and D13-2) *Pseudomonas fluorescens*(D14-2)
Hungarian cheese	*Enterococcus faecium* (H11-2)*Serratia liquefaciens*(H15-1) *Streptococcus salivarius* spp. *thermophilus* (H14-1)

pathogenicity, and alteration to organoleptic properties based on of proteases or lipases secretion are very important. For example, in the dairy industry several structures and processes (pipelines, raw milk tanks, butter centrifuges, pasteurisers, packing tools, cheese tanks,) can act as surface substrates for form biofilm at different temperatures and involve several mixed colonising species. Thus, it is essential that accurate methods to visualize biofilms in situ be set up to avoid contamination and to ensure food safety in the food industry[51].

In this study a total of 29 bacterial isolates were tested for produce biofilm formation (16 Domiaty and 13 Hangarian cheese using Tissue Culture Plate (TCP) quantitative technique. All isolates were screened for their ability to form biofilm production by TCP that measured by using Micro-plate Reader at (OD570 nm) and considered zero (0.24) according to TCP method[66]. Results of biofilm production of isolates from Domiaty cheese using method of TCP showed that 87.5% (14/16) were considered moderate biofilm formation as shown in (Table 8). Results indicated also that isolates (D11-2 and D15-1) OD570 nm were (0.405 and 0.330) respectively which considered high biofilm formation, were strong biofilm adherence. Results of biofilm production from Hungarian cheese revealed that 53.5% (7/13) were considered moderate biofilm formation as shown in (Table 9). Results showed also that 46.1% (6/13) (H6-1, H6-2, H11-1, H11-2, H13-1 and H17-2) OD_{570} nm were (0.303, 0.299, 0.307, 0.262, 0.242 and 0.362) respectively that considered high biofilm production (strong biofilm adherence).

Results in this study indicated that *Enterococcus faecium* (H11-1) bacterial strains isolated from Hungarian cheese produced strong biofilm and *Enterococcus faecalis* (H10-2) that form moderate biofilm. Different lactic acid bacterial species were isolated and identified from Domiaty cheese, (*Lactobacillus delbrueckii* subsp. *bulgaricus*, *L. casei, Lactococcus lactis* subsp. *lactis*)as reported by (Fahmy and Youssef), *L. farciminis*, *L. alimentarius*, *E. faecium, Enterococcus faecalis*[12, 67] reported that, the high rate of contamination of the examined

Table 7. Results of Blast search for DNA sequence in NCBI Genbank

Isolates	Accession No.
Raoultilla terrigena (D15-1)	MK757978
Bacillus cereus (D16-1)	MK757979
Enterococcus faecalis (H10-2)	MK757980
Enterococcus fiscalism (H11-1)	MK757982
*Serratia liquefactions (*H13-1)	MK757981
Anoxybacillus flavithermus (H17-1)	MK757984

Table 8. Biofilm formation by Tissue Culture Plate (TCP) of Domiaty cheese isolates

Number of isolates	(OD_{570} nm)	Standard	Biofilm formation	Adherence
*S. liquefaciens (*D6-1)	0.142	(0.12–0.24)	Moderate	Medium
D6-2	0.173	(0.12–0.24)	Moderate	Medium
D10-1	0.236	(0.12–0.24)	Moderate	Medium
D10-2	0.210	(0.12–0.24)	Moderate	Medium
D11-1	0.186	(0.12–0.24)	Moderate	Medium
D11-2	0.405	<0.24	High	Strong
D12-1	0.178	(0.12–0.24)	Moderate	Medium
D12-2	0.159	(0.12–0.24)	Moderate	Medium
D13-1	0.201	(0.12–0.24)	Moderate	Medium
D13-2	0.229	(0.12–0.24)	Moderate	Medium
D14-1	0.139	(0.12–0.24)	Moderate	Medium
D14-2	0.216	(0.12–0.24)	Moderate	Medium
D15-1	0.330	<0.24	High	Strong
D15-2	0.201	(0.12–0.24)	Moderate	Medium
D16-1	0.127	(0.12–0.24)	Moderate	Medium
D17-1	0.133	(0.12–0.24)	Moderate	Medium

cheese samples with *Enterobacteriaceae* is indicative for direct or indirect fecal pollution of milk used, neglecting of hygienic measures during production and handling and possible presence of enteric pathogens. Mohamed and Huang[68] reported that *E*. *faecium* and *E. facials* isolated from cheese and can be form biofilm. Kristich et al[69] reported that *E. facials* formed complex biofilm. But *E. facials* cannot form biofilm because some types of cheeses and curd cheeses incapable of biofilm formations. One of the reasons why *Enterococcus* spp. isolated from cheeses did not form biofilm could be due to the presence of sodium chloride in cheese (up to 4%) and a higher acidity of curd cheese (up to 70 SH)[70].

This study revealed that *Anoxybacillus flavithermus* (H17-2) bacterial strain isolated from Hungarian cheese produced high biofilm (strong biofilm). *Anoxybacillus flavithermus* is Gram-positive, thermophilic, and spore-forming organism that is non-pathogenic Strejc *et al*. It is a the rmophilic bacterium that is able to survive at temperatures ranging from 55 to 60°C, Khalil et al[71] and Goh et al[72] reported that *A. flavithermus* isolated from diary processing plant., and also the commonest biofilm-forming isolates are thermophilic genera in the dairy industry[43]. *A. flavithermus* spores are very heat-resistant and their vegetative cells can grow at temperatures up to 65°C with a significant increase in bacterial adhesion on stainless steel surfaces in the presence of skimmed milk. This indicates that milk positively influences these species' biofilm formation Sadiq et al[49] and Dai et al[73] reported that *A. flavithermus* isolated from water and formed biofilm.

From our study, contaminant bacteria (*Bacillus cereus* (D16-1) were isolated from Domiaty cheese and produced moderate biofilm formation. *Bacillus cereus* group may be present in a wide variety of dairy products such as milk, pasteurized milk, powdered milk, cheeses and fermented milk[74,75] reported that *Bacillus cereus* contaminated the requeijao curd cheeses. Also, isolated from feta cheese[76]. *Bacillus cereus* is a Gram-positive anaerobic or facultative anaerobic spore-forming bacterium that can grow in various environments at wide-ranging temperatures (4°C-50°C). It is resistant to chemicals, heat treatment, and radiation[40]. *B. cereus* is a frequently isolated from food and food products, such as dairy products. It secretes toxins that can cause sickness and diarrhoea symptoms in humans. *B. cereus* is responsible for biofilm formation on food contact surfaces, such as stainless-steel pipes, conveyor belts and storage tanks. It can also form floating or immersed biofilms, which can secrete a vast array of bacteriocins, metabolites, surfactants, as well as enzymes, such as proteases and lipases, in biofilms, which can affect food sensorial qualities[42]. Motility by bacterial flagella confers access to suitable biofilm formation surfaces, and is required for biofilms to spread on non-colonised surfaces. However, *B. cereus* flagella have not been found to be directly involved in adhesion to glass surfaces,

Table 9. Biofilm formation by Tissue Culture Plate (TCP) at OD570 nm of Hungary cheeseisolates

Number of isolates	(OD_{570} nm)	Standard	Biofilm formation	Adherence
H4-1	0.220	(0.12–0.24)	Moderate	Medium
H6-1	0.303	< 0.24	High	Strong
H6-2	0.299	< 0.24	High	Strong
H10-1	0.236	(0.12–0.24)	Moderate	Medium
H10-2	0.220	(0.12–0.24)	Moderate	Medium
H11-1	0.307	< 0.24	High	Strong
H11-2	0.262	< 0.24	High	Strong
H13-1	0.140	(0.12–0.24)	Moderate	Medium
H14-1	0.232	(0.12–0.24)	Moderate	Medium
H15-1	0.147	(0.12–0.24)	Moderate	Medium
H16-1	0.076	(0.05–0.12)	Weak	Weakly
H17-1	0.141	(0.12–0.24)	Moderate	Medium
H17-2	0.362	< 0.24	High	Strong

but can play a key role in biofilm formation via their motility[43]. *B. cereus* and *P. aeruginosa* showed the highest biofilm formation[77].

In our study, *Pseudomonas florescence* isolated from Domiaty cheese, and results agreement with[78-82].

From this study, *Serratia liquefaciens* (H13-1) detected in (Domiaty and Hungarian) cheeses and produced moderate biofilm formation, this results similar to Couvigny et al[83] who reported that *Serratia odorifera*was isolated from Italian cheeses and Morales et al[84] detected *Serratia* spp. in milk and cheeses. *Serratia liquefaciens* is a spoilage microorganism of relevance in the dairy industry because it is psychrotrophic, able to form biofilm, and produces thermoresistant proteases and lipases Rodrigues et al. and from milk[39].

Bacterial strain *Raoultilla terrigena* (D15-1) or *Klebsiella terrigena* obtained from Domiaty cheese that produce strong biofilm formation. These results similar to the results of Kongo and Gomes[85] who reported that *Klebsiella terrigena* and *K. ornithinolytica* strains isolated from cheddar cheese. Ogbolu et al[86] reported bacterial contamination of cheeses by *Klebsiella* species. In our study, *Streptococcus thermophilus* isolated from Hungarian cheese and had mediate biofilm formation. Our results agreement with Bassi et al[87] who reported mediates biofilm formation in dairy environments. Also, Couvigny et al[83] reported that most *S. thermophilus* strains are poor biofilm producers, mostly because they have lost these traits, consistent with their adaptation to the milk environment and selection as starters for dairy fermentations.

CONCLUSIONS

Results of identification bacteria isolates by *biomeriex* Vitek MS compact system indicated that Six of bacterial isolates from Domiaty cheese were identified as (5 *Serratia liquefaciens* (D6-1, D6-2, D14-1, D13-1 and D13-2) and one *Pseudomonas fluorescens*(D14-2)) strains. Results showed that 3 isolates of bacteria were identified as (one *Enterococcus faecium* (H11-2), one *Serratia liquefaciens*(H15-1) and one *Streptococcus salivarius* spp.*Thermophilus* (H14-1)) strains isolated from Hungarian cheese. Selected isolates were identified by16 rRNA sequencing as (*Raoultilla terrigena*(D15-1)), *(Bacillus cereus* (D16-1)), *(Enterococcus faecalis* (H10-2)), (*Enterococcus fiscalism* (H11-1)), (*Serratia liquefactions* (H13-1)), *(Anoxybacillus flavithermus*(H17-1). A total of 29 bacterial isolates were tested for produce biofilm formation (16 Domiaty and13 Hangarian cheese) using Tissue Culture Plate (TCP) quantitative technique. Results of biofilm production of isolates from Domiaty cheese showed that 87.5% (14/16) were considered moderate biofilm formation. Results indicated also that isolates (D11-2 and D15-1) OD570 nm were (0.405 and 0.330) respectively which considered high biofilm formation, were strong biofilm adherence. Results of biofilm production from Hungarian cheese revealed that 53.5% (7/13) were considered moderate biofilm formation. Results showed also that 46.1% (6/13) (H6-1, H6-2, H11-1, H11-2, H13-1 and H17-2) OD_{570} nm were (0.303, 0.299, 0.307, 0.262, 0.242 and 0.362) respectively that considered high biofilm production (strong biofilm adherence).

Miao et al[52] reported that two strategies in the industry of food: structural modification of surfaces or application of antibacterial or antibiofilm coatings[53]. Thus, several alternative products to classic disinfectants (chlorine, quaternary ammonium, etc.), such as, plant-derived antimicrobials being the compounds that display more significant antimicrobial action in shorter action times as El-Hamshary et al[13]. reported that ethanolic and ethyl acetate extracts of *Tamarix nilotica* plant showed antibacterial activity against *(B. cereus* (S1) *Staphylococcus aureus* (S2)*; Bacillus paramycoides (*S3*); Staphylococcus aureus* (S5)*; Serratia proteamaculan* (S6)*; Serratia proteamaculan* (S7) *and Serratia proteamaculan* (S9)) bacterial strains.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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This article does not contain any studies with human participants or animals performed by any of the authors.

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