Doctoral Dissertation

# MICROBIAL ANALYSIS AND BACTERIAL PROFILING OF EGYPTIAN CHEESES WITH IMPROVING THEIR SAFETY FOR HUMAN CONSUMPTION

March 2022

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# Contents

## List of Tables

## List of Figures

# Introduction

Food safety is a matter of a great and mandatory concern which reflects the level of development and humanity within the societies, many kinds of foodborne illnesses have been recognized worldwide, among them diseases of microbiological origins is given a special concern since it has been reported to be the most frequent cause responsible for more than 70% of the emergence of foodborne diseases during the last decades. The World Health Organization (WHO) has reported the global burden of foodborne disease in 2015, in its report Africa and South-east Asian regions were put on the top of the disease morbidity and mortality rate charts followed by Middle East region including Egypt (WHO, 2015).

Generally, cheese is considered as one of Time/Temperature control for safety food (TCS) (previously known as potentially hazardous food (PHF)) that facilitate the microbial growth especially if the good hygienic and safety practices are not followed. During the last ten years studies on different kinds of Egyptian cheese highlighted the prevalence of major food poisoning bacteria *E. coli* including STEC (Elhadidy and Mohammed, 2013, Hammad et al., 2009), *salmonella* (El-Baz et al., 2017, Ibrahim et. al., 2015), *Staphylococcus aureus* (El-Sharoud and Spano, 2008), *Bacillus cereus* (Enan et. al., 2012), *Campylobacter jejuni* (El-Sharoud 2009) and *listeria* spp. (Ismaiel et. al., 2014) ranging from low to high incidence rates and failing to comply with the Egyptian standards.

Studies on the microbial diversity of the traditional food has been greatly improved since the development of High throughput next generation sequencing technique (NGS) which is able to describe the overall bacterial community inhabiting a food sample rather than specific detection of one kind of bacterial species. The term Metagenomics is used to describe the recent perspective of the biotechnology art for deep understanding of the complex microbial communities useful for phylogenetic

studies, ecological studies, organoleptic characterization and enhancement of traditional foods together with investigating the pathogenic bacteria responsible for foodborne diseases which is often represent a minority among other species and sometimes go through a viable but not culturable (VBNC) state (Bergholz et al., 2014, Mayo et al., 2014., kergoulary et al., 2015). Thus identifying bacterial communities including those of potential safety impact have become available and fast.

To overcome the problems related to food safety especially those arising from bacterial contaminants, various methods of food preservation have been utilized, those included refrigeration, freezing, drying, thermal treatment, irradiation, Modified atmospheric packaging and addition of antimicrobials.

Several attempts have been studied to control pathogenic bacteria in food including cheese (Ritota and Manzi, 2020), particularly; Natural compounds that are recognized to be safe for consumption have gained the major attention due to the increased demand of new generations to eat healthy food rather than chemically preserved food, Organic Acids (OAs) are antimicrobial biopreservatives that are naturally occurring in food, their application in different food products for controlling pathogenic and spoilage bacteria has been successfully performed, furthermore; OAs have no limitation for their daily intake for human. Thus favor them as a potential solution in terms of controlling the growth of undesirable microorganisms in food matrix (Anyasi et al., 2018).

## Purpose of the Research

Taking into account the traditional method for cheese practice that may in some affect its safety for consumption, the contribution of cheese to food borne outbreaks and the shortage of information regarding the microbial composition of Egyptian cheese that contribute to organoleptic and functional properties of such a historical cheese, the aim of the present work was to:

[1] Evaluate the microbiological safety of the Egyptian cheese by culture dependent method and detect pathogenic strains by PCR method.

[2] Study the richness and diversity of Microbiota in traditional Egyptian cheeses by NGS for identifying the dominant bacterial groups and bacteria of safety and hygienic concern.

[3] Establish a control method for common contaminant of cheese using organic acids.

# Review of Literature

## History and Importance of Egyptian Cheese

Egypt as the heart of middle east area is believed to be the origin and the provider of the first archeological evidence of cheese making worldwide, processing of natural cheese which dates back to 3200 B.C. has been illustrated on old Egyptian temples and ruins' drawings (Shaker, 1983; Abou-Donia, 1991) and was also proved by a recent discovery of the oldest cheese material up to date (Fig. 1) coming out from the excavation of the tomb of Ptahmes dated to the pharaonic period, that oldest cheese sample was found between 2013 and 2014 by Cairo university and (Greco et al., 2018).

Economically, the world cheese production and consumption is obviously increasing since 2000 and is expected to continuously expand during the coming years, interestingly, Egypt is ranked as the sixth among the top 30 cheese producing countries between 2000 and 2020, and one of the two major cheese consuming countries in the Middle East and North Africa that have been reported to be the third largest consuming area in the world after EU and North America (PM Food, 2014; 2016). Cheese constitutes about 38.8% of the total dairy market value in Egypt and considered to be the major part of the dairy market where natural cheese is the largest cheese segment as reported by sales value that reached 73% of total cheese sales in 2012. Due to the pleasant taste and high nutritive value of cheese it is being consumed by all

socioeconomic Egyptian classes and all age groups as a major part of their daily diet, while being considered a main source of nutrition for people in rural areas who raise farm animals and produce cheese and other products from their milk to provide them with vital needs of Protein , fat, minerals including calcium, phosphorus and vitamins (Euromonitor 2012; Badawi, 1996; Food composition tables, 1998 ).

## Types and Manufacturing of Egyptian Cheese

Fermentation of milk into cheese is a well-known tradition since the ancient Egyptian life and was originally aimed for preservation of milk , a home-made cheese produced by farmers and housewives was an inherent custom done using traditional methods that later has been industrialized and is still being practiced by small scale producers, consequently further techniques have been developed from small scale to large scale investment where modern automation systems have been used (Shaker M., 1983; Early, 1992).

Seven main types of traditional cheeses are produced on national level including soft and hard cheeses (Fig. 2), they are mainly made form cows' or buffalos' milk or mixture of them, Romy (Ras) cheese is the most popular hard cheese type in Egypt and Arabic countries, it has a sharp and salty flavor similar to the Greek variety ''Kefalotyri'', it is produced in the shape of wheels and been cut into slices at the supermarkets, processing steps are similar to that of Gouda cheese with some few differences, Ras cheese manufacturing includes heating milk to 32° C, renneting, stirring, heating curd to 45° C over a period of 40 min(cooking), molding, brining (20% salt) for 24 hr., and dry salting for 4-5 weeks then 3-6 months of ripening and aging.

Other types of traditional cheeses consumed as soft cheeses are Kariesh, Mish, Domiati, Tallaga, Istanbuli and Baramili Cheeses (LACTIMED, 2014). Kariesh is the fresh acid cheese made from skimmed milk, in which the curd does not go any ripening process, it is usually made by traditional methods in country sides. It is preferred by consumers because it has the highest protein and lowest fat content among Egyptian cheeses (BAT4MED, 2012, Ghada et al., 2004). Traditional processing includes; milk

defatting through direct pouring in a special earthen pots and left 1-1.5 day in summer and 2-3 days in winter for the fat separation to the upper layer which is removed after that and the curd is poured onto a special mat tied at its ends together and squeezed to allow partial drainage of the whey, the mat is hung for complete drainage of whey which takes 2 or 3 days, after that the cheese is cut and dry salted (LACTIMED 2014) while under current industrial developed with commercial production of Karish cheese, different conditions may be used such as using pasteurized or homogenized milk, using starter culture and rennet (Phelan et. al., 1993) the white Karish cheese is consumed fresh or used for the production of the yellowish-brown old cheese know as Mish through pickling in microaerophilic conditions with adding of high salt, any kind of milk, nutritive substances, oriental spices, some plants and old mish as a starter, finally it is stored in warm conditions for about one year of ripening, the final taste is sharp, salty and pungent (LACTIMED, 2014), while Domiati cheese, the most popular white brined cheese in Egypt and Arab countries named also as Gebna baida, as a pickled cheese it closely resemble Feta cheese in Greece, the unique feature of Domiati cheese is the using of the milk salting technique in which (5-14%) salt is directly added to the milk at the first step of production, the cheese may be eaten fresh or more likely after keeping in salted whey for 4-8 months. The same way of production is used for making Tallaga cheese with less amount of added salt ranging from 5 to 8 % depending on the weather, the word Tallaga in Egyptian language which means refrigerator indicates that this type of cheese has to be kept in refrigerator, storage time is up to 1 month as a fresh cheese with creamy low salty taste and spreadable soft texture. Another kind of white Egyptian cheese is Istanbuli cheese which is characterized by crumbly texture and studding with Jalapeno chilies resulting in its remarkable spicy flavor. It is quite salty where salt starter culture is added to fresh pasteurized milk. (LACTIMED, 2014. Mehanna & Rashed, 1990, Shehata et.al., 1995, El-Kholy, 2005).

However traditional practices of cheese making have been thought to influence some health benefits and contribute to the original sensory characterization including cheese flavor (Montel et al., 2014), such a condition may threaten the public health

since raw milk is being used without pasteurization increasing the microbiological hazard of the cheese.

## Control of Undesirable Bacteria in Cheese

Cheese consumption is a common culture worldwide, but the possibility of being contaminated with spoilage and pathogenic microorganisms has imposed the use of preservatives in cheese manufacturing for the purpose of maintaining both safety and quality parameters. Natural preservatives that are recently accepted by modern consumers are being widely used in different kinds of cheeses all over the world, antimicrobials that are Generally Recognized as Safe (GRAS) for food preservation incudes plant extracts (Ritota and Manzi, 2020), essential oil, lysosomes, nisin and natamycin are some examples (Jalilzadeh et al., 2015).

Several investigations has been performed to control pathogenic bacteria in Egyptian cheese. Chitosan was used to inhibit the growth of *Staphylococcus aureus* and *Escherichia coli* in Karish cheese (El-Dahma et al., 2017). EOs of Zingiber officinale (ginger), Syzygium aromaticum (clove), and Thymus vulgaris (thyme) were fortified to white cheese for the control of Methicillin-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Ahmed et al., 2021).

Organic Acids are among food additives that are permitted to be used in various kinds of food. Beside being recognized as GRAS additives, unlike Eos they are easily to be manipulated due to their solubility in water that can make them an easy and safe intervention for controlling undesirable bacteria in food (Anyasi et al., 2018).

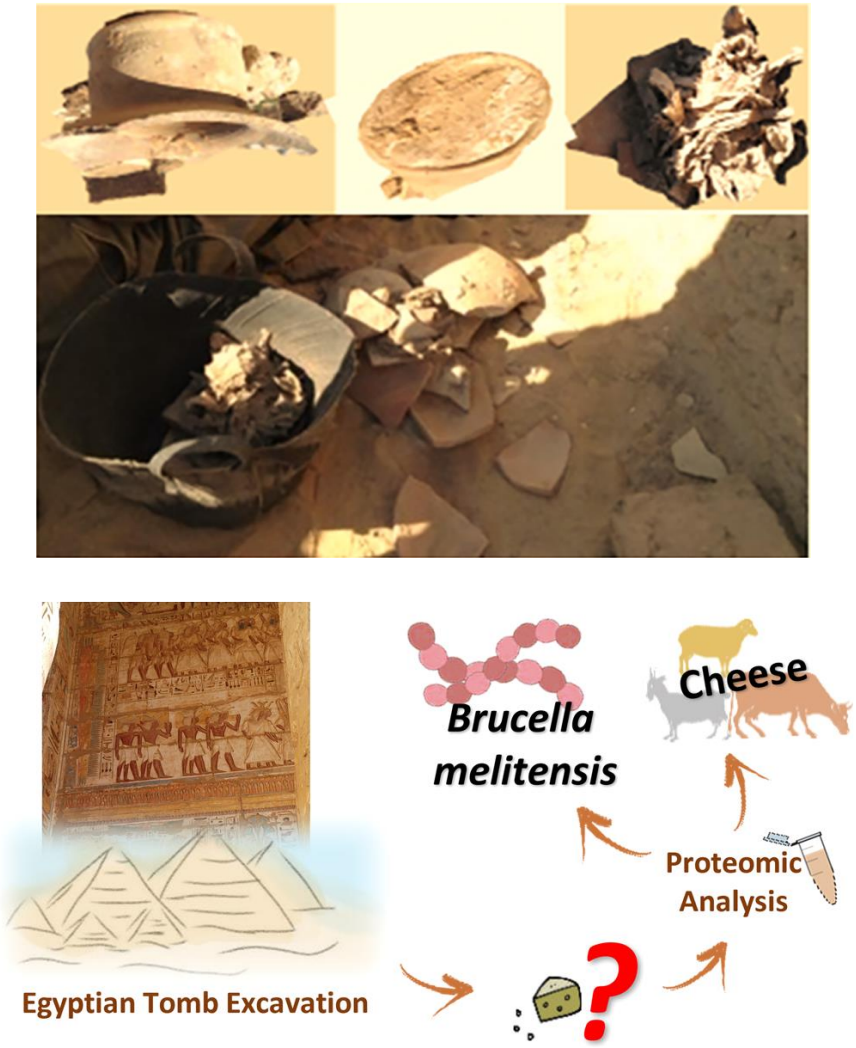



Figure 1 Jar and canvas discovered inside the tomb of Ptahmes, Mayor of Memphis during the XIX dynasty (Greco et al., 2018).


Figure 2 Types of Egyptian cheeses

# Chapter 2. Analysis of the Microbial Community in Egyptian Cheese

## Section 1: Microbiological safety of Egyptian Cheese

### Introduction

Microbiological associated hazards can gain access into cheese through different routes from farm to fork including processing, handling and storage, beside the possible presence of high microbial load in the used raw milk, thus can lead to food poisoning which is considered as a major public health problem and may rise to loss of business. However many cases of food poisoning are not serious and can be improved without any treatment, still some sever cases are being reported, hospitalized and/ or required antibiotic treatment (Kotsonis and Burdock, 2008). According to the Center of Disease Control (CDC) 6, 839 foodborne disease outbreaks were reported in the united states in 2016, resulting in 14,259 illnesses, 875 hospitalizations, 17 deaths, and 18 food product recalls where the most common etiological bacterial agent was *Salmonella* causing 33% of outbreaks followed by Shiga toxin-producing *Escherichia coli* accounting for 6% of outbreaks (CDC, 2018). In a retrospective study done at the Poison Control Center, affiliated to Ain Shams University, Cairo, Egypt, 1748 individual cases were reported during the first half of the year 2010 while the most affected group of people were young adults (36%) followed by school children (22%) (Abd-Elhaleem and Abd-Elkarim, 2011).

Cheese is a perfect media for microbial growth as defined by the U.S Food and Drug Administration (FDA) Food Code, most cheeses are potentially hazardous foods (PHF) due to the pH range, water activity and high protein content (FDA, 2009). Dairy products were reported as one of the most common food caused outbreaks in U.S. in 2016 (CDC, 2016).some kinds of cheese were able to support the growth of the pathogenic bacteria *L. monocytogenes*, *Salmonella* spp., *E. coli O157:H7 and S. aureus* (Leong et al., 2014), and with absence of good hygienic and safety practices traditional Egyptian cheese could fail to comply with both international and Egyptian standards (Sayed et al., 2011). several studies have reported that various kinds of pathogenic bacteria may be isolated from cheese in Egypt including *E. coli, Salmonella spp.*

*S.aureus, B.cereus,* and *Listeria spp.* with presence of high number of total coliform and total colony counts (El-Etriby, 2017; Hassan and Gomaa, 2016). For the sake of the safety of human being consuming cheese, in the first chapter of this study the microbiological safety of 6 types of popular Egyptian cheeses was assessed by counting of (Total Aerobic bacteria, *Enterobacteriaceae, E.coli* and lactic Acid bacteria) and detection of major pathogenic bacteria (*Salmonlla spp*. and *Listeria spp*.) by culture methods, after that PCR was used to confirm the presence of pathogenic strains, furthermore the salt concentration was measured for each sample.

## Materials and Methods

### Sampling

A total number of 19 different commercial cheese samples representing 6 types of popular Egyptian cheeses were obtained from 5 geographical zones located in Cairo and Kalyobia governorates of Egypt. Samples were categorized into two major groups; soft cheese (5) named as Kariesh, Mish, Domiati, Tallaga, and Istanbuli, and hard cheese type (1) named Romy or Ras cheese. Most samples were obtained from supermarkets (13) while other samples were obtained from local market/street vendors (6), samples were cooled and transported to the laboratory and stored at 4° C before microbiological analysis. Information on cheese type, location and market are presented in Fig. 3 and Table 1.

### Microbiological Analysis

### Enumeration of viable Bacterial counts

Ten grams of each cheese sample were added to 90 mL of saline solution containing 0.85% sodium chloride and homogenized in a stomacher bag for 30 sec at 230 rpm using a stomacher 400-T homogenizer (Seward, London, UK), same saline solution was also used for serial dilution up to $10^7$ CFU/g of each homogenized sample**.** One milliliter of each dilution series $10^3$-$10^1$ CFU/g were inoculated onto appropriate Petrifilm count plates (3M, St. Paul, USA). For counting of total volatile bacterial (TVC), Enterobacteriaceae (EB) and *E.coli*, 3M-aerobic count plates (AC), Enterobacteriaceae count plates (EB/VRBG**)** and *E.coli*/Coliform count plates (EC) were used respectively, plates were incubated at 37° C and viable bacteria were counted after 24 h in case of Enterobacteriaceae (EB) and 48 h in case of aerobic count and *E.coli* (AC and EC). Man Rogosa Sharp Media (MRS) agar plates (MRS, Oxoid, Hampshire,

UK) were used for counting lactic acid bacteria (LAB), plates were inoculated with the serially diluted samples $10^7$-$10^1$ and incubated at 30°C for 72 h. Each dilution was inoculated onto duplicate of plates to obtain the mean value for all kinds of enumerated bacterial strains.

**Detection of *Listeria* spp and *Salmonella* spp.**

**Culture dependent method**

For culture based microbial detection the ISO 11290 method was used for isolation of *Listeria* spp. on PALCAM agar media (Merck, KGaA, Darmstadt, Germany). Briefly, 10 g of each cheese sample were primary enriched in 90 mL of half-Fraser broth (Oxoid) which is a selective media with half concentration of selective agent and incubated at 30° C for 24 h, then a secondary enrichment by inoculation of 9 mL of Fraser broth (Oxoid) with 1 mL of the culture obtained from primary enrichment was performed and incubated at 30° C for 24 h, 0.1 mL of each obtained culture was plated on PALCAM agar solid media (2 plates for each culture) using spread-plate method and also streaked on 2 other PALCAM agar plates. Plates were incubated at 30° C and examined after 48 h for the presence of typical *listeria* spp. colonies.

For isolation of *Salmonella* spp., 10 g of each cheese sample were homogenized in 90 mL of Trypticase Soy Broth "TSB" (BD, Becton Dickinson, New Jersey, USA) as a pre-enrichment media, incubated over night at 37°C, then 1 mL of the enriched broth was transferred to 9 mL of Hajna Tetrathionate Broth (EIKEN, Tokyo, Japan.) incubated overnight at 37°C, after that each sample was streaked on 2 plates of Deoxycholate Hydrogen sulphide Lactose Agar (DHL) media (EIKEN) media, incubated at 37°C for 24 h and examined for *Salmonella* spp.

Suspected colonies were then sub-cultured on the specific solid media mentioned before for each strain with the same incubation conditions, single colonies were picked, inoculated into TSB broth and incubated overnight, then 10 µL were transferred to new TSB agar for purification, incubated overnight and vortexed. Finally 1 mL of the purified cultures were transferred to sterile Eppendorf tubes, centrifuged at 11700 x*g* rpm for 3 min, supernatants were discarded and pellets were stored at
-20° C to be used for DNA extraction in the next step.

**PCR method**

Polymerase Chain Reaction (PCR) was used as a confirmatory test after the culture dependent method for the detection of pathogenic strains. DNA was extracted from the cell pellet obtained in the previous step using Nucleo Spin extraction kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. Briefly, STET (Saline/Tris/EDTA/Triton* X-100 Solution) lysis buffer solution was added and incubated at 37° C for 1 h, followed by cell lysis using proteinase K and incubation at 56° C overnight. DNA was bound to silica membrane in the column after the addition of B3 buffer (incubated at 70° C for 10 min) and100% ethanol respectively. Double washing using BW and B5 buffers followed by adding BE (elution buffer) resulted in the purified DNA that was used as a template for PCR reaction. DNA concentration was then measured using spectrophotometer and was adjusted to be 5 ng/µL.

For specific detection of *Listeria* spp., a designed pair of forward and reverse primers, BE-LiSAll (F: 5 GAA MGA ATG AAA GCG CTA CGA GAY AAR GT 3, R: 3 TCC CCA CCW GCT AAA TAR TGR CTT TC 5) were used which produce a product size of 463 bp as described by Phraephaisarn et al. (2017), PCR reaction was performed in GeneAmp thermal cycler (PCR System 9700, Applied Biosystems) in a 50 µL reaction mixture for each sample contained 5µL 10xExTaq Buffer, 4µL dNTPs, 2µL of each primer, 0.25 Ex Taq polymerase, 5µL template DNA and 31.75µL of DW. Amplification was run for 40 cycles with the following condition; initial incubation at 95°C for 5 min, denaturation at 95°C for 10 sec, annealing at 60°C for 30 sec, elongation at 72°C for 30 sec, and final extension at 72 °C for 3 min.

While for *Salmonella* spp. a set of primers described by Rahn *et al*. (1992) of about 284 bp with the following nucleotide sequence (F; invA 139. M90846 GTG AAA TTA TCG CCA CGT TCG GGC AA, R; invA 141 TCA TCG CAC CGT CAA AGG AAC C) were used for the specific detection of *Salmonella* spp. targeting the *invA* gene located on the pathogenicity island 1 of that species (Collazo and Galán, 1997). The reaction was carried out for a 50 µL reaction mixture contained 5µL of 10xExTaq Buffer, 4µl dNTPs, 5µL of each primer, 0.25 Ex Taq polymerase, 5µL template DNA and 25.75 µL of DW. The PCR reaction was run for 30 cycles, cycle condition was as follow; initial incubation at 95°C for 5 min, denaturation at 95°C for 15 sec, annealing at 65°C for 45 sec, and final extension at 72° C for 20 sec using GeneAmp thermal

cycler. For both examined strains 5µL of aliquots of the PCR products were further analyzed by agarose gel electrophoresis.

### Measurement of salt concentration

Salt concentration of cheese samples was measured using a digital salt meter (LAQUA Twin Salt-22, Horiba, Japan), 10 g of each cheese sample were homogenized in a stomacher bag with 10 mL of distilled water (DW), 1 mL of the homogenized cheese samples were used for measuring after calibration of the salt meter with standard solution according to the manufacturer's instructions.

## Results and Discussion

### Viable Bacterial Count

In the present study, microbiological characterization of cheese has been studied first by culture dependent method, a total number of 19 cheeses were analyzed representing 6 different types including soft and hard cheeses named (Karish, Domiati, Tallaga, Mish, Istanbuli and Romy) were collected from five geographical origins and different market types (street vendors/local, supermarkets), general results of microbial counting indicated that the overall cheese microbial counts showed significant bacterial load including total aerobic count, Enterobacteriaceae and E.coli (Table 1).

Total Aerobic counts (TVC) were more than 5.15 log CFU/g in 13 out of 19 cheese sample up to 7.35 log CFU/g in hard type cheese (Romy H2), while lowest values were recorded in soft type cheese that was 2.51 log CFU/g in both Mish cheese S12, and in Istnabuli cheese S15 and 2.57 log CFU/g in Domiati cheese S6. Monitoring the total aerobic count of the cheese is the first indicator usually used by most researchers addressing the microbiology of the food including cheese, for example; El-Etriby, 2017 has studied the microbiology of some soft and hard cheeses starting with measuring the TVC, her result showed a range of 2.4 log CFU/g to 6.5 log CFU/g which is relatively same like our finding ( 2.51 to 7.35 log CFU/g ), other suggested that their result between 1.6 and 5.2 log CFU/g of TVC was close to the guidelines of Egyptian standard (2001) (Ghada et al, 2004) inconsistent with our and other studies where

it could reach 7.35 log CFU/g in the present study and log 8.3 CFU/g in some samples of Domiati and Kariesh cheeses (Hassan and Gomaa, 2016) . while our study also found a high incidence (68%) of cheeses with > 5.15 log CFU/g for TVC, in a worth case > 6.2 log CFU/g mean value were present in 100% of all tested Egyptian cheeses suggesting an inferior quality of the marketed cheese (Heikal et al., 2014).

*Enterobacteriacae* counts were highly variable ranging from 2.04 to 6.66 log CFU/g in Domiati cheese S15 and Kariesh S2 respectively with only two samples were free from *Enterobacteriaceae* (Domiati S6 and Mish S11). This family represents a wide range of bacterial pathogens including important food born, opportunistic and food spoilage pathogens that gives a special concern to limit its coun in food staff. Since this family can be eliminated by normal pasteurization of milk, its presence in cheese samples may be related to using raw milk for cheese processing, process failure, poor handling or after processing contamination suggesting that the Enterobacteriaceae is an important indicator for the overall good manufacturing practice (GMP) under which the cheese is being produced in addition to its role as an indicator of fecal contamination usually comes from employees' hands and poor sanitary conditions (Baylis et al., 2011)

Beside the safety concern of that group, some members of *Enterobacteriaceae* are implicated in flavor changes of cheese by producing specific enzymes break down the milk protein (Casein), these enzymes can't be inactivated neither by Ultra-heat (UHT) nor high temperature-short time (HTST) treatment, others can cause color change and pigmentation of cheese (*Serrati* spp.) Regarding Coliform group as an important member of Enterobacteriaceae has the same implications beside its ability to ferment milk protein (lactose) producing gas causing early bowing and holes in cheese (ICMSF, 1998), studies on coliform count in Egyptian cheeses has revealed a range from 3.39 to 4.41 log CFU/g (Heikal et al., 2014), from 1.3 to 4.3 log CFU/g (El-Etriby,2017), from 2 to 7.2 log CFU/g (Hassan and Gomaa, 2016) and some times reaching 7.8 log CFU/g which is exceeding the acceptable limit set by Egyptian Guidelines 2001, our results also confirmed the high possibility of the Egyptian cheese to contain a considerable amount of *Enterobacteriaceae* group with possible contribution to gastrointestinal illness and cheese defects.

As explained before, since Enterobacteriaceae includes a diverse large groups, it may be a good indicator of overall GMP rather than specific kind of spoilage or contamination caused by particular pathogen, and because the Enterobacteriaceae is usually inhabitant in the gastrointestinal tract of animals and humans suggesting its importance as an indicator for fecal contamination, particularly *E. coli* strains are the most common bacteria considered for this purpose (Baylis et al., 2011) . In the present study *E. coli* were detected in 12 out of 19 cheese samples (68%) with wide concentration range between 1.00 and 4.41 log CFU/g, regarding the concentration, the Egyptian standard (2005) requires the *E. coli* don't exceed 1 log CFU in one gram of cheese, suggesting that about 47.4 % of cheeses (9) analyzed in the present study were unable to comply with the regulations regarding *E. coli* count, while other 7 samples were totally free from it including all types of Mish cheese S11, S12, S13, one Kariesh cheese S2, one Domiati cheese S6 and two Istanbuli cheeses S15, S16 and 2 samples S3 and H2 were found to be containing *E. coli* but not exceed the limit suggested by Egyptian standard. this finding is relatively higher than other findings regarding the incidence of *E. coli* in Egyptian cheese where other researchers has isolated them from 51.5% (Ghada,Z.A.A et al., 2004) , 48% (El-Etriby, 2017) and 26.7% (Heikal et al., 2014) of soft cheese samples,12 to 28% in Domiati and Kariesh cheeses (Hassan and Gomaa, 2016), with slightly higher concentration up to 4.8 log CFU/g by Ghada et al. (2004) than reported in the present study.

Results in the present study is also confirming the knowledge that the presence of Enterobacteriaceae in food is not necessary to be considered as a fecal contamination (Baylis et al., 2011), although cheese samples number S2, S12, S13, S15 and S16 were contaminated with considerable amount of Enterobacteriaceae (6.66, 2.9, 2.51, 5.7 and 5.49) log CFU/g respectively, they were totally free form *E. coli* usually indicating fecal contamination, in another study by El-Etriby (2017); the incidence of coliform in Egyptian hard cheese reached 68% of examined 25 samples with a mean value of 2.5 log CFU/g while all samples were also free from true fecal type of *E.coli*, this emphasize the necessity of testing *E. coli* as a single parameter determining the sanitation and hygienic level in production and handling of cheese, furthermore measuring the extent of awareness and training of the food handlers. On the other hand, when Enterobacteriaceae were not detected (sample no. S6), result of *E. coli* count must be also zero in the same sample as shown in Table.1, in conclusion; it can be said that "incidence

of *E. coli* in food material is surely proves the presence of Enterobacteriaceae but not the opposite."

Lactic acid bacteria (LAB) were isolated and counted using MRS agar, results revealed their presence in all cheese samples except soft Mish cheeses S12, S13 and S15 at concentrations comprised between 3.45 and 8.33 log CFU/, specifically all 16 positive cheeses had a high concentration of LAB except only 2 cheeses (S11 and S16) that recorded a count of 3.6 and 3.45 respectively. LAB are responsible for the organoleptic characteristics of cheese and are known for its health benefits derived from its probiotic nature through various mechanisms, the functional and technological role of various species of LAB favor their presence in fermented food like cheese. The high count of LAB in Egyptian cheeses which is common to fermented products may have played an important role for the control of pathogenic bacteria (*Listeria* spp. and *Salmonella* spp.) (Losito et al., 2014)

**Detection of pathogenic strains (*Listeria* spp and *Salmonella* spp.) by culture dependent method**

Results of selective culture method for isolation of *Listeria spp.* (Table.1) showed that 8 samples (S2, S6, S8, S10, S11, S14, H1 and H2) were suspected to be contaminated since typical colonies have been observed on PALCAM agar media, while only 4 samples were showing typical *Salmonella spp*. colony on DHL agar (S1, S7,S10 and H3) reflecting possible contamination with Salmonella spp.

**Confirmation of suspected colonies of pathogenic Strains (*Listeria spp* and *Salmonella spp.)* by PCR method**

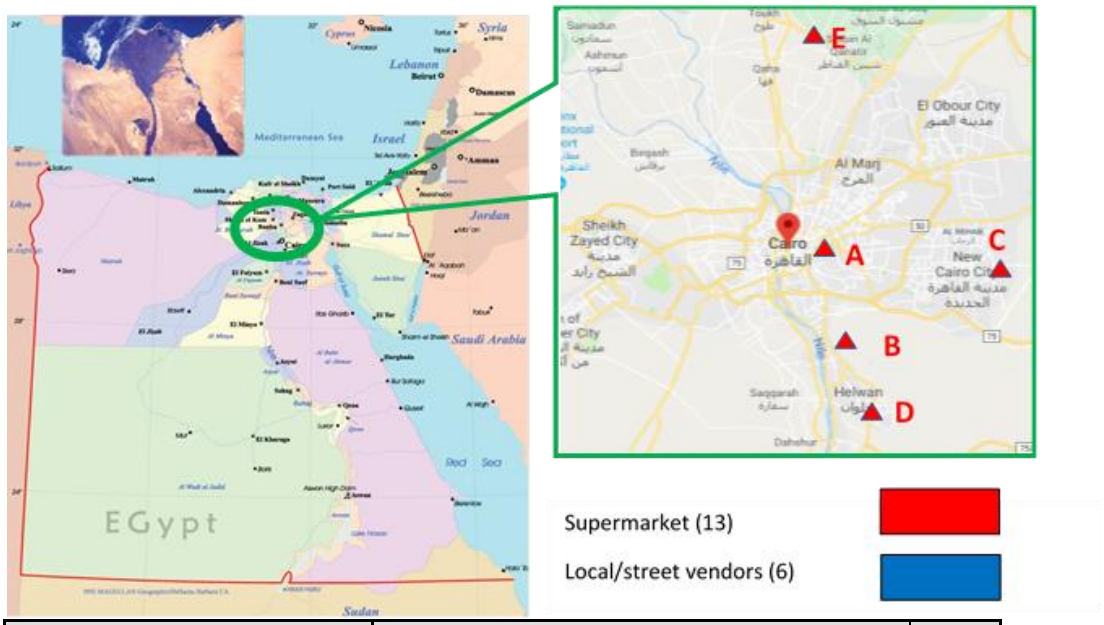
Confirmation of the results obtained by culture-dependent method for detection of *Listeria spp.* and *Salmonella spp.* has been done by PCR for all suspected samples using single colonies, however as shown in Fig. 4 and Fig. 5 all samples were negative for both strains. This finding is in contrast with the frequent previous reports of detection of pathogenic bacteria in Egyptian cheese including *Listeria* spp. and *Salmonella* spp. (El-Baz, 2017; Ibrahim et al., 2015; Ismaiel et al., 2014; Ghada et al., 2004). However, those results were mainly based on traditional culture dependent methods, despite using specific growth factors, microorganisms of similar

growth condition may confuse the final result. Our result confirms the well-known fact that PCR is the most accurate and superior technique for detection of microorganisms in food outweighs other traditional culture dependent methods. Thus researchers cannot rely only on culture-dependent methods for the final confirmation of the presence of food pathogens specially those of possible implication of outbreaks results in severe illness like *listeria* spp. and *Salmonella spp*. (CDC, 2016; 2018).

**Salt Concentration**

Salt concentration varied according to the cheese type ranging from 0.48% in Karish cheese up to 12% in Domiati cheese (Table 2, Fig.6), low salt concentration was generally reported in both Karish and Tallaga Cheeses from a concentration of 0.48% to 3%, Moderate concentration in Hard Type Romy cheeses H1, H2 and H3 (1.62% to 5%) highest salt concentration was found in Mish, Istanbuli and Domiati cheeses.

In this chapter, Viable cell counts of TVC, *Enterobacteriaceae*, *E.coli* and LAB were performed using classical culture method specific for each species, further more contamination with pathogenic *Salmonella* spp. and *Listeria* spp. was investigated by combination of culture-dependent and culture-independent method for confirmation. Results indicated that the overall microbial load of Egyptian cheese was significant despite the freedom from pathogenic species, viable cell counts suggested that cheese were of poor quality that reflects a bad image of the overall GMP during cheese production particularly poor personal hygiene with possible contamination of fecal material. Moreover, possible contribution of the counted bacteria to cheese defects (color change, gas production, holes) and spoilage should not be neglected.




| Location | | Ras | Karies. | Mish | Domiat. | Tallaga | Istanbu: | Totlal |
|---|---|---|---|---|---|---|---|---|
| Down Town/cairo | A | | | | | | | 5 |
| Maadi/Cairo | B | | | | | | | 5 |
| New Cairo | C | | | | | | | 4 |
| Helwan/Cairo | D | | | | | | | 1 |
| Kalyobia | E | | | | | | | 4 |
| | Total | 3 | 3 | 3 | 3 | 4 | 3 | 19 |

*Figure 3 Distribution of samples according to location and market type*

**Table 1** Microbiological characterization of Egyptian cheese including viable counts (log CFU/g) and presence of pathogenic strains (*Listeria* spp and *Salmonella* spp.)

| no. | Cheese Name | Cheese Type | Source | Location | Viable Bacterial Count (Log CFU/g) | | | | *Pathogenic strains* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | TVC | EB | *E. coli* | LAB | *Listeria spp.* | *Salmonella spp.* |
| 1 | Karish | S1 | local market | D | 5.85 | 4.72 | 1.18 | 7.01 | n.d | ND |
| 2 | Karish | S2 | Supermarket | B | 6.06 | 6.66 | 0.00 | 6.80 | ND | n.d |
| 3 | Karish | S3 | local market | E | 5.77 | 5.81 | 1.00 | 7.59 | n.d | n.d |
| 4 | Domiati Cheese | S4 | Supermarket | A | 5.68 | 4.73 | 0.70 | 6.85 | n.d | n.d |
| 5 | Domiati Cheese | S5 | Supermarket | B | 6.69 | 3.58 | 3.54 | 5.86 | n.d | n.d |
| 6 | Domiati Cheese | S6 | Supermarket | C | 2.57 | 0.00 | 0.00 | 6.01 | ND | n.d |
| 7 | Tallaga | S7 | Supermarket | A | 6.83 | 5.08 | 5.03 | 7.48 | n.d | ND |
| 8 | Tallaga | S8 | local market | E | 6.94 | 5.81 | 4.41 | 8.33 | ND | n.d |
| 9 | Tallaga | S9 | Supermarket | B | 6.87 | 5.02 | 2.00 | 8.03 | n.d | n.d |
| 10 | Tallaga | S10 | Supermarket | C | 6.38 | 2.54 | 1.40 | 7.51 | ND | ND |
| 11 | Mish | S11 | local market | A | 4.02 | 0.00 | 0.00 | 3.60 | ND | n.d |
| 12 | Mish | S12 | Supermarket | C | 2.51 | 2.90 | 0.00 | 0.00 | n.d | n.d |
| 13 | Mish | S13 | local market | E | 5.15 | 2.51 | 0.00 | 0.00 | n.d | n.d |
| 14 | Istanbuli | S14 | Supermarket | A | 4.89 | 2.04 | 1.78 | 7.37 | ND | n.d |
| 15 | Istanbuli | S15 | Supermarket | B | 2.51 | 5.70 | 0.00 | 0.00 | n.d | n.d |
| 16 | Istanbuli | S16 | local market | E | 3.51 | 5.49 | 0.00 | 3.45 | n.d | n.d |
| 17 | Romy | H1 | Supermarket | A | 6.26 | 2.37 | 3.04 | 5.89 | ND | n.d |
| 18 | Romy | H2 | Supermarket | B | 7.35 | 5.90 | 1.00 | 6.52 | ND | n.d |
| 19 | Romy | H3 | Supermarket | C | 6.56 | 2.10 | 1.60 | 6.62 | n.d | ND |

Values of bacterial count are mean of duplicate measurements.

n.d; no typical colony was detected, ND; at least one typical colony was detected by culture method but confirmation by PCR method revealed negative detection,

H: Hard Cheese type, S: Soft cheese, A: Down town of Cairo, B: Maadi/ cairo, C: New Cairo, D: Helwan/Cairo, E: Kalyobia

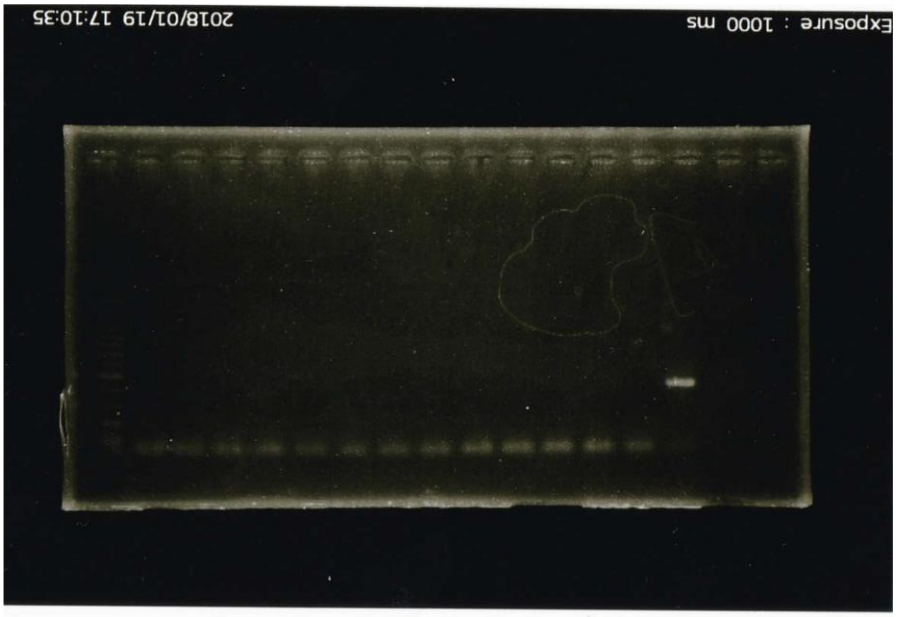



*Figure 4 Results of PCR detection of* Listeria *spp. in different types of traditional Egyptian cheeses.*

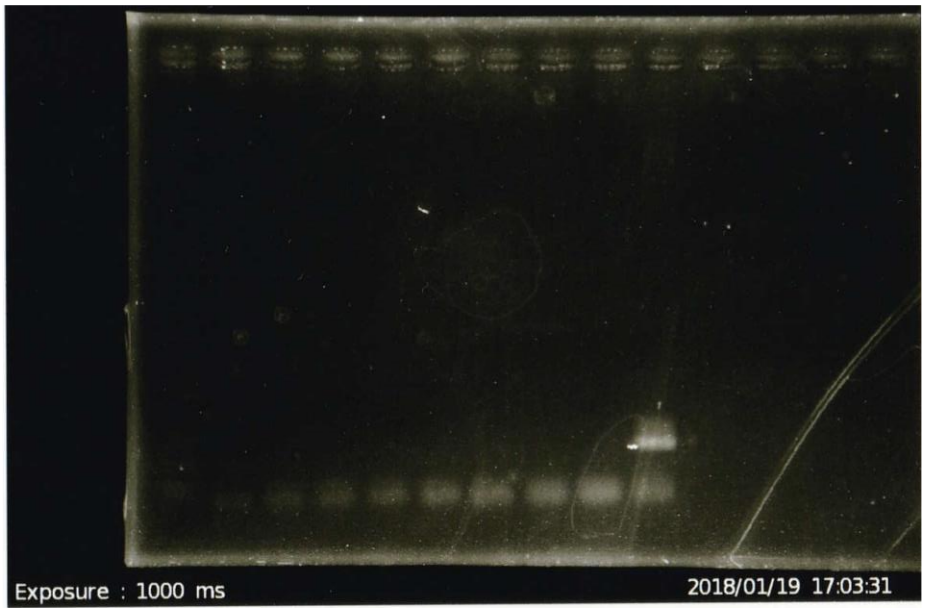



Figure 5 Results of PCR detection of *Salmonella* spp. in different types of traditional Egyptian cheeses.

**Table 2. Salt concentration in different types of traditional Egyptian cheeses (%).**

| Sample no. | Type | Cheese Name | Salt Concentration (%) |
|---|---|---|---|
| 1 | Soft type Cheese | Kariesh Cheese | 3.00 |
| 2 | | Kariesh Cheese | 0.48 |
| 3 | | Kariesh Cheese | 1.54 |
| 4 | | Domiati Cheese | 12% |
| 5 | | Domiati Cheese | 6.40 |
| 6 | | Domiati Cheese | 6.40 |
| 7 | | Tallaga Cheese | 1.20 |
| 8 | | Tallaga Cheese | 1.48 |
| 9 | | Tallaga Cheese | 1.28 |
| 10 | | Tallaga Cheese | 1.48 |
| 11 | | Mish Cheese | 11.60 |
| 12 | | Mish Cheese | 3.80 |
| 13 | | Mish Cheese | 10.00 |
| 14 | | Istanbuli Cheese | 1.20 |
| 15 | | Istanbuli Cheese | 8.00 |
| 16 | | Istanbuli Cheese | 11.00 |
| 17 | Hard Type | Romy Cheese | 1.62 |
| 18 | | Romy Cheese | 5.00 |
| 19 | | Romy Cheese | 2.20 |

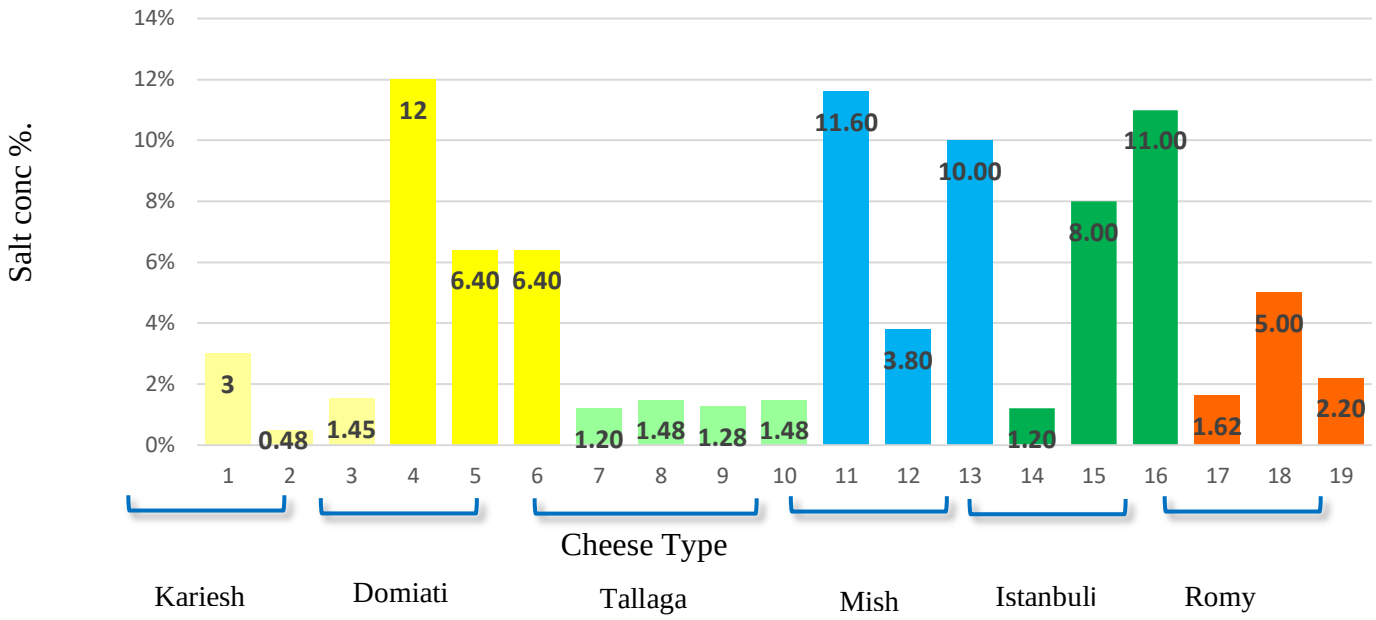



*Figure 6 Comparison between salt concentrations in different types of traditional Egyptian cheeses.*

# Section 2. Biodiversity of Bacterial Communities in Egyptian Cheese

## Introduction

Recently the Next Generation Sequencing has greatly outpaced the first sequencing techniques introduced by Maxam-Gilbert and Sanger techniques as the first generation due to its high productivity being able to generate thousands and up to millions sequence reads in one sample "High-throughputs Sequencing" HTS that had made a great evolution in genomic science in a way that could shortcut an effort over 15 years of work required for sequencing of the first human genome in 2001 to only one day where 45 human genome sequencing can be achieved since the release of HTS in 2014. (illumina, 2014). The impact of the NGS on food microbiology has as well been remarkably revolutionized, NGS analysis of different types of food including milk and dairy products have been successfully carried out achieving the deep insights into the composition of the target food microflora (Marino et al., 2017; Ercolini et al., 2012; Li et al. 2018; Escobar-Zepeda et al. 2016). Pyrosequencing has also been used to investigate the bacterial communities and dynamics of bacterial populations present during the manufacture and ripening of traditional cheeses, including Danish raw milk cheeses (Masoud et al., 2010), the Polish cheese Oscypek (Alegría, A., et al., 2012), water buffalo Mozzarella (Ercolini et al, 2012), artisanal Irish cheeses (Quigley, L. et al., 2012), Latin-style cheeses ( Lusk, T.S. et al., 2012) and Iranian Liqvan cheese (Ramezani et al., 2017) where new bacterial types have been identified for the first time in traditional cheese.

Since cheese is well-known of its richness and diversity of different microbial communities that is been transferred from the environment to ripened cheese ( Montel et al., 2014), researchers in the food sector have highlighted the importance of using HTS as a powerful technique useful not only for characterization of microbiota of cheese and dairy related products with close relevance to dairy industry responsible for the organoleptic and other physio-chemical characteristics of cheese, but also for understanding different factors affecting

the composition of these microbiota including seasonal variation, animal feed, milk source and other environmental factors (Jonnala et al., 2018).

Few studies on the Biodiversity of Egyptian cheese have been conducted, where some researchers has relied on classical culture-dependent method for analyzing the pathogenic bacteria, and used the culture-independent technique PCR-temporal temperature gel electrophoresis (TTGE) for only LAB in Karish cheese ( Awad, 2016), TTGE has also been used by El-Baradei et al. (2005) in kariesh cheese and combined with PCR-denaturing gradient gel electrophoresis (DGGE) by the same author in 2007 for the other popular Egyptian domiati, the results confirmed the interest of using culture-independent methods for characterization and understanding of other Egyptian cheeses with using of more advanced techniques. However, PCR-DGGE method is able to detect the main bacteria of cheese same like pyrosequencing, the former could not detect the minor bacteria in cheese where pyrosequencing showed deeper understanding of the bacterial microflora (Masoud et al., 2011), thus so far no metagenomic analysis using NGS for Traditional Egyptian type cheese has been reported. In the present study and for the first time the metagenomic technique that has become the main technology for understanding the biodiversity of traditional cheese has been used to study six types of popular Egyptian cheeses including soft and hard cheeses.

## Material and Methods

### Sample Preparation

All 19 samples used in the present study have been analyzed by NGS, 10 g of each cheese sample were added to 90 mL of 0.85% saline solution and homogenized in a stomacher bag for 30 sec, 1 mL of the homogenized cheeses were transferred into sterile Eppendorf tubes, centrifuged at 11700 x*g* rpm for 3 min, supernatants were discarded and pellets were stored at -20° C for microfloral analysis.

### DNA Extraction

One hundred microliter of Achromopeptidase enzyme was added to each sample pellet prepared from the $10^1$ diluted cheese sample as described before for more efficient DNA

extraction of gram-positive bacterial strains, then the mixtures were incubated at 55° C for 15 min. DNA was extracted from the samples using Nucleo Spin extraction kit according to the manufacturer's instructions.

**NGS workflow**

Amplicon sequencing of the 16S rRNA gene for the amplification of V3 and V4 regions across multiple bacterial species was performed, the 4 basic steps of NGS workflow including library preparation, cluster generation, sequencing and data analysis were carried out by Bioengineering Lab. Co. (Atsugi, Japan), Briefly; library preparation was done using a two-step polymerase chain reaction (PCR) method

First PCR was performed to measure the DNA quality in 25 cycles of PCR (GeneAmp thermal cycler) with the following condition; initial incubation at 94°C for 2 min, denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, elongation at 72°C for 30 sec and final extension at 72° C for 5 mins. each 40 µL of reaction mixture contained 4 µL of 10xExTaq Buffer, 3.2 µL dNTPs, 2 µL of each primer (Table 3), 0.4 Ex Taq polymerase, 2 µL template DNA and 26.4 µL of DW. PCR cycles were increased to 30 for unclear bands with the same conditions. After that PCR products were purified using the AMpure purification kit according to the manufacturer's instructions (Beckman Coulter, Brea, California, USA) in three stage of selective binding of PCR products to paramagnetic beads and separation of the beads with a magnetic field, washing the beads to remove contaminants and eluting the purified PCR products from the magnetic beads, then DNA concentration was measured by spectrophotometric means at absorbance of 260 nm.

Second PCR was performed by Bioengineering Lab. Co, the individual DNA was tagged in a ten-cycle PCR reaction using the primer sequences in Table 3, PCR condition was as follow; 10 cycles of initial incubation at 94°C for 2 min, denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, elongation at 72° C for 30 sec and final extension at 72° C for 5 mins, 10 µL reaction mixture contained 1 µL of 10xExTaq Buffer, 0.8 µL dNTPs, 0.5 µl of each primer, 0.1 Ex Taq polymerase, 2 µL template DNA and 5.1 µL of DW.

Sequencing was done using Illumina MiSeq instrument according to the manufacturer's instructions (Illumina, San Diego,CA.USA). obtained sequences were clustered into operational taxonomic units (OTUs) according to 97% identity cut-off using the QIIME workflow script. Alpha diversities of the bacterial microflora in each sample were expressed using Shannon diversity index (Shannon, 1948). Furthermore, Principle component analysis (PCA) was conducted on OTUs, the calculations for PCA were done using the R "prcomp" function and plotted using the "plot" function, and bacterial communities were clustered according to cheese type.

## Results and Discussion

Bacterial communities of cheese samples were assessed by next generation sequencing, a large number of sequences have been clustered into many OUT groups. Overall 7868 OTUs were detected in the present study considering the communities from all samples, observed OTUs significantly varied among cheese types. Read numbers, observed OTUs and Shannon diversity index of each sample are shown in Table 4. the lowest number was observed in Karish cheese1 (101) while the highest observed OTUs were recorded in Istanbuli cheese 15 (875). Shannon diversity index has indicated a diverse microbiota in Egyptian traditional cheeses ranging from 0.3 in Karish cheese 2 that had the lowest Shannon diversity index within all types of examined cheeses, to 3.27 in Istanbuli cheese 14 that had the highest diversity index as shown in table.4 with an average of 2.8, a relatively similar average 2.7 was recorded in Italian cheesemaking brines (Marino et al., 2017)while higher diversity index was observed in Irish cheese with an average of 3.8 and 3.7 in soft and hard cheeses respectively. Despite the relative abundance of bacterial OTUs and observed diversities, our results suggested that salt concentration had no effect on the diversity of the bacterial communities since high diversity was observed in both law salt (Ch 14, Ch 18 and 19) and high salt cheeses (Ch 4, 11 and 16) that was in agreement with results obtained by Marino et al. (2017) who suggested that salinity concentration is not the only factor associated with microbial diversity since unexpectedly, he found that highest diversities were associated with the highest salinity in cheese brines in contrast with the fact that salinity has a selective property on microbial composition of food.

In total 8 bacterial phyla including dominant and minor groups were observed in Egyptian Cheeses with different abundance within cheese samples as shown in Table 5 and Figure 7. Cheese bacterial communities were dominated by members of the *Firmicutes* (78%) and *Proteobacteria* (18.7%), followed by *Actinobacteria* (1.9%), and *Bacteroidetes* (1%), those four phyla are commonly found in cheese environment (Quigley et al., 2012; Marino et al., 2017; Riqueleme et al., 2015), a fifth phylum with a minor prevalence 0.3% was also detected "*Cyanobacteria*". The relative abundance of these five phyla was quite variable among cheese samples while 3 phyla (Figure 7).

Seventy families were detected in the present study, where 25 of them were present at abundance > 1% in at least one sample. The most abundant bacterial families were *Streptococcaceae*, *Lactobacillaceae*, *Leuconostocaceae*, *Enterococcoceae*, *Staphylococcaceae*, *Xanthomonodaceae*, *Pseudomonadaceae*, *Oceanospirrillaceae*, *Vibrionaceae*, and *Enterobacteriaceae* that were present in most examined cheeses (Figure 8). Many of the dominant families were widely distributed across the samples, but their abundance within each cheese community was variable, somehow related to the type of cheese, bacterial families in Egyptian cheese seemed to be more abundant comparing to Italian cheese brine and Pico Portugalian cheese, all families dominated in the later two studies were shared between Egyptian cheese, furthermore Egyptian cheese appeared to be more rich on the family level where larger number of families have been detected. the presence of *Bifidobacteriaceae* is usually limited to salt concentration up to 5%, however this family has been detected in 9 samples of Egyptian cheese where 4 of them had a high salt concentration range between 6.4 and 12% which was supposed to supress their growth, same finding was observed in Italian cheesemaking brine where the author did not expect their presence since brine had a very high salt concentration up to 19.57%, it was suggested that they had been adapted to high salinities with the possibility to be used as a probiotic candidates in high salt food including cheese (Marino et. al., 2017; C. Riqueleme et al., 2015; De Castro-Cislaghi et al., 2012). The incidence of the family *Xanthomonodaceae* in Egyptian cheese as the sixth predominant family was unexpected and never been related to the cheese environment, since members of this family are usually characterized as environmental organisms suggesting possible contamination from the surrounding environment during cheese processing and handling. This group of thermophilic

bacteria should be effectively eliminated from cheese since they are heat-resistance thus might prevent their destruction by pasteurization (LaSala, P.R. et al., 2007; Liu, S. et al., 2011)

Analyzing the microbial diversity to the deeper taxonomic assignment showed that a total of 101 different genera were identified, 39 of which were present at least at 1% in at least one cheese sample (Fig. 10). 10 genera (*Lactobacillus*, *Staphylococcus*, *Enterococcus*, *Leuconostoc*, *Lactococcus*, *Streptococcus*, *Lysobacter*, *Enterobacteriaceae*; g, *Pseudomonas* and *Acinetobacter*) composed the core microbiome of cheese, since they were found in at least 14 cheese samples out of 19 regardless to their variable prevalence within samples. *Lactobacillus* and *Lysobacter* were found in all cheeses. *Macrococcus* and *Chryseobacterium* were quite widespread in cheese (12 out of 19). Minor genera have been also identified including the *Tetragenococcus* that was found in 3 samples with a concentration of 28 to 29.6%

The abundance of different bacterial genera showed a significant variation depending on cheese type as shown in Fig. 9. *Lactobacillus* spp. and *Enterococcus* species were significantly more prevalent in hard Romy chees, while their relative abundance was significantly lower in soft cheeses. Whereas *Streptococcus* was more prevalent in soft Karish and showed low prevalence in Tallaga cheese comparing to other cheeses, *Lactococcus* species dominated in all soft cheeses than hard cheese. *Enterobacteriaceae* were wide spread in all cheese types with the higher percentage observed in Karish soft type cheese (no.3) in a percentage of 11.8. *Streptococcus* spp. was highly significant in Kariesh soft cheese no.1 and no.2 with a concentration of 88.9% and 96.2% respectively. *Tetragenococcus* was found in Domiati and Istanbuli cheeses, *Leuconostoc* and *Marinomonas* were only found in Tallaga cheese with very high abundance.

Pyrosequencing is basically performed in basis to assign the OTUs on the genus level, however within the observed OTUs in the present work 102 were identified at the species level whereas some pathogenic species were detected such as *Staph aureus* and *Bacillus cereus*. *Staph aureus* gains a particular interest within the dairy environment since it might cause food born intoxication by *Staphylococcal* enterotoxins (SEs), SEs that represents the major virulence of this species are strong heat resistant able to survive in dairy products even after pasteurization (Argudín et al., 2010), *S. aureus* has been occasionally detected in Italian cheese brine and dairy products including Egyptian cheeses (Marino et al., 2017; El-Baradei et al., 2007; Sayed et al.,

2011; El-Etriby, 2017). unfortunately, patients suffer from *Staphylococcal* food poisoning may have difficulty for antibacterial treatment, since this strain is known to carry resistance gene against methicillin antibiotic, several studies have reported the incidence of Methicillin resistance *S. aureus* (MRSA) in Egyptian cheeses and other cheeses, suggesting that dairy environment is a good reservoir for those strains (Malt; Kamal et al., 2013; Al-Ashmaway et al., 2016; Adame-Gomez et al., 2018). The second alarming pathogenic species that was occasionally detected "*Bacillus cereus*" has a great relevance to food poisoning in dairy industry however its primary source is soil, pasteurization causes it sporulation, surviving spores are considered the source of food poisoning while the two different types of *Bacillus* food poisoning (emetic and diarrheal) are caused by different types of toxins. The development of psychrotrophic strains in the dairy industry has led to increasing surveillance of *B. cereus* in recent years (Granum and Lund, 1997). It is well noted that this pathogen has frequently been isolated from Egyptian cheeses in previous studies (Hassan and Gomaa, 2016; Enan et al., 2012)

Coagulase negative staphylococci (CNS) such as *S. equorum*, *S. Sicuri* and *S. epidermidis* were observed on the species level, the two later species have the ability to form biofilm that may persist for long time on food processing surface (Marino et al., 2011), in addition to the opportunistic nature of *S. epidermidis* making it a major cause of nosocomial infection, that pathogen is a normal inhabitant on the skin and may also originate from subclinical mastitic cows (Otto 2009; Thorberg et al., 2009), on the other hand *S. equorum* that was frequently associated with cheese environment Marino et al., 2017) is considered a functional species plays a role in the organoleptic characterization and color development of the cheese (Mounier et al., 2006) as well as exhibiting an antibacterial activity against *Listeria monocytogenes* on cheese surface ( Bockelmann et al., 2017).

**Karish Cheese (Ch1, Ch2, Ch3)**

Traditional Karish cheese showed the lowest read numbers, OTUs and diversity index within all examined Egyptian cheeses, which is probably due to its freshness and simple manufacturing without addition of starter culture or ripening process, The bacterial microbiota on genus level of Karish cheese are shown in Fig.11 , Kariesh cheeses were dominated by lactic acid bacteria including *Streptococcus*, *Lactobacillus* and *Lactococcus* that were in accordance with the finding obtained by TTGE analysis in Kariesh cheese (El-Baradei et al., 2005) as well

as other kinds of cheeses including Italian, Danish and Portugalian cheeses (Dalmasso et al., 2016; Masoud et l., 2011; Riquelme et al., 2015). Particularly *streptococcus* genus showed distinctive abundance and dominance in two Karish cheeses that constituted 88.9% and 96.2% in Ch 1 and Ch2 respectively while *Lactococcus* dominated in the third sample S3 (53.1%), furthermore, Ch3 showed a significant increase in both OTUs and Diversity index comparing Ch1 and Ch2. Awad, 2016 suggested that Lactobacillus and *Lactococcus* species play the main role in the fermentation of Kariesh cheese as a result of TTGE analysis (Awad, 2016), however our study showed that *Streptococcus* might has the highest contribution, probably the variation in the analysis method may explain that difference, other non-lactic acid bacteria (*Lysobacter*, *Pseudomonas*, *Enterobacteriaceae*, *Macrococcus*, *Aeromondacae* and *Acinetobacter*) were detected in most or all Karish cheeses, different species of *Enterobacteriaceae* have been reported before by DGGE analysis, other bacterial genera that were occasionally detected in Karish cheese in the present study included *Staphylococcus*, *Acetobacter*, *Corynebacterium*, *Bacillus* and *Planococcaceae*. Our study demonstrated that Karish cheese manufacturing that does not involve ripening process is mainly controlled by members of *Streptococcaceae*, with the contribution of members of *Gammaproteobacteria*, *Streptococcus* dominated in two cheese samples, but *Lactococcus* in the third one, interestingly; that third sample showed high abundance of *Staphylococcaceae* including *Staphylocccus* and *Macrococcus* suggesting that either *streptococcus* was not a good competitor with *staphylococcaceae* (Janek et al., 2016), or that 3 samples were not produced under a standardized condition.

**Domiati Cheese**

The bacterial microbiota on genus level of Domiati cheese are shown in Fig.12. Domiati Cheeses (Ch4, Ch5 and Ch 6) which showed high sequence reads, OTUs and Diversity index, were very rich on the genus level, they contained approximately all the detected genera in the present study except only 5 occasional genera were absent in Domiati cheese (*Marinomonas*, *Vibrio*, *Streptophyta*, *Bacillus* and *Candidatus Portiera*). This cheese has been described by its richness of bacterial communities that were classified into 3 main groups, group one composed of dominant bacterial species *Leuconostoc mesenteroides, Lactococcus garvieae, Aerococcus*

*viridans, Lactobacillus versmoldensis, Pediococcus inopinatus/Macrococcus caseolyticus,* and *Lactococcus lactis subsp. lactis* that may play the main role in the fermentation and organoleptic properties of Domiati, The second group is composed of frequently encountered bacterial species. These bacteria belonged to both LAB species, e.g., *Leuconostoc citreum, Lactobacillus casei, Lactobacillus johnsonii, Staphylococcus thermophilus, E. faecalis, E. faecium/Leuconostoc pseudomesenteroides*, and to non-LAB species, e.g., *Vibrio spp., K. kristinae, C. variabile, K. rhizophila, Arthrobacter spp./B. tyrofermentans, plus numerous species of* coagulase-negative *staphylococci*. While third group consists of occasionally encountered bacterial species, e.g., *Lactococcus raffinolactis, Acinetobacter lwoffii, Staphylococcus lentus, S. chromogenes, Enterobacter cloacae*, and *Klebsiella oxytoca*. The author suggested that bacteria of the second and third groups may have a secondary activity in the fermentation process of Domiati cheeses, but the origins and potential roles of these as well as the bacteria of the first group would need further investigation (El-Baradei et al., 2007). Despite the species defined in the previous study and the absence of Vibrio and bacillus genus in our study, the three groups were shared with samples of Domiati cheese in the present work, we also suggest to add members of other novel bacterial genera that were not previously detected in Domiati cheese including *Lysobacter, pseudomonas, Chryseobacterium, marinilactibacillus, Weisella, Enhydrobacter* and *Planococcaceae* since they were found in all examined Domiati cheeses, as well as *Tetragenococcus, Corynebacterium, halanaerobium* and *Kocuria* that were found in 2 out of 3 samples, and finally two genera that were detected in only one cheese (*Micrococcaceae* and *Alteromondales*). Unexpectedly; significant difference in the dominance bacteria has been observed between the 3 Domiati cheeses, in Ch4 *Tetragenococcus, Lactobacillus, Streptococcus* and *Lactococcus* were dominated (29.4%, 26.9%, 11.2% and 7.9%) , Ch5 was dominated by members of *Lactococcus, Lactobacillus, Streptococcus* and *Staphylococcus* (31.6%, 19.2%, 9.2% and 7.7%) while Ch6 was dominated by pediococcus 37% followed by a member of *Proteobacteria* (*Salinivibrio* 33.3%) and *Staphylococcus* 16.8%, and in the same time Ch6 showed a significant lower diversity index (1.87) comparing to Ch4 (2.83)and Ch5 (3.08), the *Tetragenococcus* spp. that predominated in Ch4 is most probably survived due to its halotolerant nature (Marino et al., 2017) since this cheese showed the highest salt concentration (12%) not only between Domiati cheeses but also all other types of cheeses,

However this is the first time to be reported in Egyptian cheese and has never been reported in Italian cheese but found in their brines for the first time by Marino et al. (2017), while been reported in an older study on smoked polish cheese (Marino et al., 2017; Algeria et al., 2012). The prevalence of *Pedioccocus* spp. in Ch6 as a member of LAB suggests that they play the main role in the fermentation of this sample in addition to its functional property as a probiotic that can confer a possible health benefit due to the Bacteriocin production (Martino et al., 2013; Dobson et al., 2012). The salt-loving nature of *Salinivibrio* spp. may explain their high prevalence since halophilic bacteria are adapted to grow in moderate and high salt concentration environment suitable for production of lipolytic and proteolytic salt-stable enzymes (Gorriti et al., 2014; AlJohny, 2015).

**Tallaga Cheese (Ch7, Ch8, Ch9, Ch10)**

The bacterial microbiota on genus level of Tallaga cheese are shown in Fig. 13. The low salt white soft Egyptian Tallaga cheese appeared to have the second lowest value of diversity index and OUT number after Kariesh Cheese within all examined cheese types, analyzing the cheese microbial composition on the genus level by NGS showed that 3 samples (Ch7, Ch9 and Ch10) were dominated by *Leuconostoc* spp. in a percentage of 50.1, 60.9 and 47.1% respectively, followed by *Marinomonas* in Ch7 (43.9%), *Vibrio* in Ch9 (10.3%) and *Lactobacillaceae* in Ch10 (29.3%), while Ch8 had a different dominance pattern where *Lactococcus* dominated (46.1%) followed by *Pseudomonas* (29.6%). Interestingly; *Marinomonas*, and *Vibrio* were only found in Tallaga soft cheese within all types of examined cheeses, *Marinomonas* represented 43.9% of the total genera in one of Tallaga cheeses (S7) and 6.6% in two other Tallaga cheeses (S9 and S10). While *Vibrio* represented 10.3% of the total genera in Ch9 and 0.1% in Ch10. Furthermore; unidentified genera of *Gammaproteobacteria* constituted 7% and 0.3 % of the cheese microflora of Ch9 and Ch 10 respectively.

**Mish Cheese (Ch11, Ch12, Ch13)**

The bacterial microbiota on genus level of Mish cheese are shown in figure 14. This yellowish-brown old cheese together with Istanbuli cheese revealed the highest diversity indexes in the present study with an average of 2.8, it was found to have high read numbers and OTUs as well,

just like previous described cheeses differences in dominance bacteria on the genus level was observed between the 3 cheeses from the same type, in particular; Ch11 was dominated with *Lactococcus* spp. (18.6%), *Lactobacillus* (14.9%), non-lactic acid bacteria (*Lysobacter* 14.1% and *Pseudomonas* 13 %), and *Streptococcus* (11.1%), while other subdominant bacterial genera were *Enterobacteriaceae*, *Bacillus*, *Leuconostoc*, , *Corynebacterium*, *Staphylococcus* and *Psychrobacter* in a percentage of 7.7%, 4.6%, 3%, 2.7%, 2% and 2% respectively, 3 bacterial genera were occasionally detected lower than 1% (*Streptophyta*, *Enterococcus* and *Tetragenococcus*). Ch12 was dominated by *lysobacter* spp. where it showed the highest abundance between all the 19 cheese samples (46.7%), the following abundance genus in this samples were *Lactococcus* (10.3%) and *Acetobacter* (5.9%), the later was significantly detected only in this sample while was occasionally present in other 8 samples in a very low percentage (less than 1%), surprisingly; this sample recorded the highest number of observed OTUs in the genus level considering the OTUs of more than 1% in at least one sample (in total =39) showed in Fig. 10, 32 out of 39 genera have been found in this sample while in other cheeses 7 to 28 genera were observed, furthermore unique genus was accidently detected only in the same sample named *Candidatus Porteira* in a percentage of 1.4%. this genu is related to what is called "Endosymbiotic bacteria" that shows a specific interaction within their hosts which is usually arthropods and insects, species of this genus is recognized as the primary endosymbiont of the cotton whitefly " a highly invasive agriculture pest worldwide that damage a wide range of vegetables and crops" (Raina et al., 2015), to the best of our knowledge this the first report of this genus in cheese, unfortunately they probably owe their prevalence to failure of pest control program in the processing environment specially that this sample was obtained from supermarket and had a different processing style from other mish cheeses since it was mostly made by mixing with other kinds of processed cheeses in a factory level and not from Karish cheese like on the farm level in other 2 samples, another possibile origin may be the vegetable frequently added to Mish cheese during its ripening (LACTMED, 2014) that might come from infected plant which have not gone under an effective inspection procedure as a part the raw material's supplier control system , however from an optimistic point of view the detection of this kind of bacteria known to be neither culturable nor genetically manipulatable (Kikuchi, 2009) is considered as a success point for the NGS technique highlights the importance of using

such technique by food processors as a one tool able to evaluate and monitor quality, safety and overall food safety management system on industrial level. In Mish cheese sample number 13, bacterial genera including *Lactococcus, Streptococcus, Leuconostoc* and *Salinivibrio* were dominated (27.7%, 21.3%, 8.7% and 8.1% respectively) followed by *Pediococcus* 6.7%, *Corynebacterium* 5.2% (the highest value in all 19 samples), *Staphylococcus* 4.9% and others.

However, pyrosequencing results revealed that LAB were the dominant bacteria in 2 Mish cheeses and significantly detected in the third one, this was not consistent with the result of culture-dependent method in chapter 1 of this study where LAB have not grown at all on MRS agar in 2 Mish Cheese samples (Table 1). Since LAB are not among the common bacteria that enter in VBNC state (Nicolò and Guglielmino, 2012) we cannot claim their unviability to this case. Although LAB are typically microaerophiles that is the same condition of Mish cheese production together with the high salt concentration are two selective factors for LAB growth and functioning in this cheese, a possible challenge that LAB might have confronted with the Mish cheese environment is the acid stress which is a self-imposed stress leading to cell death due to the prolonged exposure to acid stress resulted from accumulation of lactic acid by LAB over one year of ripening in Mish cheese (LACTMED, 2014; Even et al., 2002).

In General Mish cheese was quiet unique within other kinds of Egyptian cheeses since a number of bacterial genera were of unique incidence and more prevalence than other cheeses, those included *Aerococcus, Corynebacteria, Aetobacter, Streptophyta, Bacillus, Psychrobacter* and *Candidatus Porteira* that might be related to the specific characteristics and condition of this historical cheese being produced under microaerophilic condition, ripened and aged for about one year in traditional earthen ware pots in addition to richness of ingredients and salt (LACTMED, 2014).

**Istanbuli Cheese (Ch14, Ch15, Ch16)**

Bacterial microbiota on genus level of Istanbuli cheese are shown in Fig. 15, *Lactococcus, Streptococcus, Aerococcus* and *Staphylococcus* dominated in Ch 14 in a percentage of 18.8%, 14.4%, 11.6% and 8.2% respectively, followed by other *streptococcaceae* 7.5%, *Pediococcus* 6.9%, and *Enterobacteriaceae* 6.8%. other 21 genera were minor in a prevalence less than 5% (in total 28 detected genera) including but not limited to *Lactobacillus,*

*Pseudomonas, Enterococcus, Macrococcus, Chryseobacteria, Acinetobacter, Marinilactibacillus, Halanaerobium, Enhydrobacter* and *Alteromondalis*, the later 3 genera which were occasionally detected in other cheeses but showed their highest prevalence in this sample compromising 2.8%, 1.1% and 2.1% respectively from the whole microbial composition of Ch14. While Ch15 and Ch16 had lower number of OTUs (21 and 20), Ch15 was dominated with *Lactobacillus* (35.9%), *Tetragenococcus* (29.6%) and *Staphylococcus* (17%), while Ch16 was dominated with *Streptococcus* (31.6%), *Tetragenococcus* (28%), *Lactococcus* (12.6%) and *Lactobacillus* (11.6%). Interestingly Istanbuli cheeses 15 and 16 shared the highest prevalence of *Tetragenococcus* (28% to 29.6%), and *Kocuria* as a minor (1.1% to 1.6%) with Domiati cheese 4, those 3 samples (Ch4, Ch15 and Ch16) recorded a high salt concentration (12%, 8 %and 11%) that might explain their prevalence due the halotolerant nature of *Tetragenococcus* spp., this species is known for its probiotic property that might be a potential source probiotic for application in salted food (Marino et al., 2017). *Kocuria* spp. has been isolated from milk and fermented dairy products in India, their psychotropic nature may be the reason for their growth in dairy products including cheese (Patil, 2019), this genus was isolated from desert soil in Egypt (Li et al., 2006), however their growth in cheese is not favored since they are a possible source of chees spoilage.

**Romy/Ras cheese (Ch17, Ch18, Ch19)**

The bacterial microbiota on genus level of hard Romy cheese are shown in figure 16, this cheese showed the second highest shannon diversity index within other cheese types in an average of 2.7, observed genera were an average of 20, all samples of this cheese CH17, Ch18 and Ch19 were dominated with *Lactobacillus* spp. that constituted 54.8%, 31.3% and 32.2% respectively of the cheese bacterial microflora, followed by *Staphylococcus* (22.6%) in Ch17, *Enterococcus* (30.7%) in Ch18 and *Streptococcaceae* (19.4%), *Lactococcus* (16.3%) and *Leuconostoc* (13.3%) in Ch19, minor species such as *Psseudomonas*, *Enterobacteriaceae*, *Chryseobacterium*, *Acinetobacter*, *Weissella* and *Propionibacterium* were detected in all samples with different abundances, the later two species were significant in Romy cheese comparing to other cheese types. *Weissella* species has been reported before in Mexican cheese which suggests that it may have a minor contribution to cheese flavor (Escobar-Zepeda et al.,

2016). It is almost linked to the wooden surface used for cheese manufacturing and ripening of Romy cheese, the source of this species in Italian cheese was also thought to be wood (Settanni et al., 2012). On species level *Propionibacterium* Acnes was identified, this environmental contaminant that might cause skin wounds as an opportunistic pathogen has been also detected in cheese environment before (Marino et al., 2017; De Filippis et al., 2014).

**Principal Component Analysis (PCA)**

In the present work PCA analysis was used to cluster the bacterial communities according to the cheese type and salt concentration, the result of PCA analysis was highly consistent with the finding of NGS analysis on the genus level that were explained previously in details. PCA is shown in Figure 19, most samples were grouped according to cheese type, particularly the highest homology was seen in Kariesh cheeses 1 and 2, except for Ch3 obtained from local market that was not grouped with the same cheese type. Tallaga cheeses 7, 9 and 10 were grouped together as well with exception of Ch8 (local market), two cheese out of 3 from the same type in case of Mish, Istanbuli and Romy cheeses were also grouped together with relatively lower homology comparing to grouped samples of Karish and Tallaga cheese. Despite the frequent ungrouping of one sample with same type of cheese (Ch3 in Karish, Ch8 in Tallaga, Ch 12 in Mish (obtained from supermarket), Ch14 in Istanbuli and Ch 19 in Romy) the majority of samples tended to group together according to its type except for Domiati cheese Ch4, Ch5, and Ch6 that were not grouped at all, probably the high salt concentration of Ch4 that was almost double Ch5 and Ch6 seemed to group this sample with other high salt samples of Istanbuli cheeseCh16 and Ch15 and in the same time Ch14 that was not grouped had a low salt concentration, interestingly this group (Ch4, Ch16 and Ch15) recorded the highest prevalence of *Tetragenococcus* spp. as explained before which suggests that high salt concentration was decisive in their case for some species selectivity and grouping pattern, same pattern was observed in Italian cheesemaking brine (Marino et al., 2017). Another factor that might affected the composition of bacterial microbiota as seen in PCA analysis is the market type, i.e., the ungrouped samples obtained from local market in Karish and Tallaga cheeses, while in case of Mish cheese only samples from local market were grouped together, that pattern of grouping based on market type was observed in grouping of Ch11, Ch3 and Ch13 that were obtained from

local market despite different cheese type. Regardless of small number of cheeses showing belonging to each group It can be suggested that the main factors affecting the diversity of microbiota in Egyptian cheese are cheese type including manufacturing process that is supposed to be uniform, Salinity and market type that reflects the manufacturing style (artisanal or industrial), other factors that were not analyzed in the present work includes the manufacturing process with possible addition of starter culture and adjunct bacteria, milk type and heat treatment, production date and season, pH and possible adulteration (Quigley et al., 2012). Moreover, some specific equipment used in the Egyptian culture for making cheese such as the use of special mat for drainage of Karish cheese and earthen pots for ripening and aging of Mish cheese needs further investigation for their contribution to composition of cheese microflora.

It is well observed that geographical region had no relevance with the cheese biodiversity, same observation was noted previously when rind samples of cheeses produced in different location showed similar pattern of microbial cluster (Wolfe et al., 2014).

The pattern observed in Domiati cheeses was exceptional and unexpected since none of them showed any similarity to others, the variability in manufacturing process across factories might be the main driver for this difference in the diversity of bacterial microbiota. In deed consistency of the manufacturing process and production condition usually show great homology in the microbial composition of cheese (Delcenserie et al., 2014) this degree of homology was also observed in brine samples collected from same plant that tended to group together (Marino et al., 2017).

From a practical and realistic point of view it is impossible to find 100% homology in the bacterial community inhabiting a cheese sample, many factors can affect the composition of cheese microbiota, on the farm level; factors such as milking animal (Quigley et al., 2012), teat skin microflora (Frétin et al., 2018), type of animal feed (Giello et al., 2017) could contribute to the composition of cheese microflora, during production; milk pasteurization/raw milk (De Filippis et al., 2014), PH, salt content, degree of ripening, ingredients added including spices and herbs (*Quigley* et al., 2012), wooden equipment (Escobar-Zepeda et al., 2016). have a significant effect on the selection of microbes, and indeed it might differ between cheese producers according to the production process and the use of starter bacteria or not (Dalmasso

et al., 2016)., while after sale; handling, storage practice and time would also affect, even though, trials to minimize these variabilities have been employed on large scale industries and with using standard starters, still variation between different batches of the same production date can be observed (O'Sullivan et al., 2015).

In chapter 2, pyrosequencing of different types of Egyptian cheese was able to detect the biodiversity of the bacterial ecosystem providing a deep insight into the composition of dominant, subdominant and minor bacterial communities starting form phylum level until family, genus and some species level. Egyptian cheese had significant results in terms of richness and diversity, despite the wide range of functional bacterial genera that have been detected, Egyptian cheese seemed to be a reservoir of spoilage and pathogenic bacteria as well, thus affecting the cheese shelf life and puts the population under a significant risk for food borne diseases. Furthermore, clustering of OTUs based on cheese type in PCA analysis revealed that some Egyptian cheeses appeared to have a degree of homology in the manufacturing process while others did not cluster in one group suggests that standardized production of cheese is not well implemented, in particular the greatest homology was observed in low-salt cheeses (Kareish and Tallaga), while Domiati high salt cheeses did not show any clustering based on cheese type, this finding suggests that the secondary factor that may be selective for bacterial composition is salt concentration.

Table 3. Primers used for NGS analysis in first and second PCR reactions.

| *Name of primer* | *Sequence (5 →3)* |
|---|---|
| *1st →27F mod_MIX** | ACACTCTTTCCCTACACGACGCTCTTCCGATCT-NNNNN- AGRGTTTGATYMTGGCTCAG |
| *1st → 338R_MIX** | GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-NNNNN- TGCTGCCTCCCGTAGGAGT |
| *2nd F* | AATGATACGGCGACCACCGAGATCTACAC-Index2-ACACTCTTTCCCTACACGACGC |
| *2nd R* | CAAGCAGAAGACGGCATACGAGAT-Index1-GTGACTGGAGTTCAGACGTGTG. |

Table 4. Total raw read numbers, OTUs and Shannon index of cheese samples sequencing with NGS.

| SAMPLE NUMBER | RAW READ NUMBER | OBSERVED OTUS | SHANNON INDEX |
|---|---|---|---|
| **CHEESE-1** | 43,157 | 101 | 0.78 |
| **CHEESE-2** | 63,240 | 167 | 0.3 |
| **CHEESE-3** | 60,936 | 287 | 1.97 |
| **CHEESE-4** | 62,267 | 732 | 2.83 |
| **CHEESE-5** | 67,989 | 529 | 3.08 |
| **CHEESE-6** | 75,962 | 384 | 1.87 |
| **CHEESE-7** | 67,065 | 112 | 1.05 |
| **CHEESE-8** | 58,890 | 196 | 2.08 |
| **CHEESE-9** | 72,169 | 190 | 1.53 |
| **CHEESE-10** | 74,589 | 210 | 1.67 |
| **CHEESE-11** | 59,146 | 399 | 2.83 |
| **CHEESE-12** | 70,726 | 403 | 2.65 |
| **CHEESE-13** | 67,905 | 583 | 3 |
| **CHEESE-14** | 61,873 | 468 | 3.27 |
| **CHEESE-15** | 70,114 | 875 | 2.46 |
| **CHEESE-16** | 63,296 | 603 | 2.57 |
| **CHEESE-17** | 59,096 | 320 | 2.15 |
| **CHEESE-18** | 62,775 | 421 | 3.19 |
| **CHEESE-19** | 65,082 | 495 | 2.82 |
| **AVERAGE** | 64,54 | 393 | 2.3 |
| **TOTAL** | 1,226,277 | | |

Table 5: Relative abundance of OTUs at the Phylum level.

| Bacterial Phyla / Samples | Firmicutes | Proteobacteria | Actinobacteria | Bacteroidetes | Cyanobacteria | Spirochaetes | Thermi | TM7 | Others |
|---|---|---|---|---|---|---|---|---|---|
| Total | 78 | 18.7 | 1.9 | 1 | 0.3 | 0 | 0 | 0 | 0.1 |
| Cheese 1 | 98.7 | 1.3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Cheese 2 | 98.8 | 1.1 | 0 | 0.1 | 0 | 0 | 0 | 0 | 0 |
| Cheese 3 | 78.3 | 19.3 | 2.3 | 0.1 | 0 | 0 | 0 | 0 | 0 |
| Cheese 4 | 84.6 | 6.9 | 4.1 | 3.8 | 0 | 0 | 0.2 | 0.1 | 0.2 |
| Cheese 5 | 86.1 | 11.7 | 0.7 | 1.4 | 0 | 0 | 0 | 0 | 0 |
| Cheese 6 | 61.9 | 36.8 | 0.7 | 0.5 | 0 | 0 | 0 | 0 | 0.1 |
| Cheese 7 | 55.3 | 44.6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Cheese 8 | 60.6 | 36.5 | 2.1 | 0.9 | 0 | 0 | 0 | 0 | 0 |
| Cheese 9 | 73.5 | 26.3 | 0 | 0 | 0 | 0 | 0.1 | 0 | 0 |
| Cheese 10 | 90.5 | 9.4 | 0 | 0 | 0 | 0.1 | 0 | 0 | 0 |
| Cheese 11 | 55.9 | 39.7 | 3.1 | 0 | 0.9 | 0 | 0 | 0 | 0.3 |
| Cheese 12 | 34.1 | 60.3 | 1.1 | 0.5 | 3.7 | 0 | 0.1 | 0 | 0.2 |
| Cheese 13 | 76.9 | 16.3 | 5.9 | 0.3 | 0.4 | 0 | 0.1 | 0 | 0.1 |
| Cheese 14 | 79.5 | 17.6 | 1.2 | 1.3 | 0 | 0 | 0 | 0 | 0.4 |
| Cheese 15 | 90.3 | 4.9 | 3.1 | 0 | 1.2 | 0 | 0 | 0 | 0.4 |
| Cheese 16 | 91.2 | 3.9 | 2.9 | 1.8 | 0 | 0 | 0.1 | 0.1 | 0 |
| Cheese 17 | 92.4 | 3.2 | 2 | 2.4 | 0 | 0 | 0 | 0 | 0 |
| Cheese 18 | 81.1 | 11.9 | 5.5 | 1.4 | 0 | 0 | 0 | 0 | 0 |
| Cheese 19 | 91.9 | 3.7 | 1.4 | 2.8 | 0 | 0 | 0 | 0.1 | 0.1 |

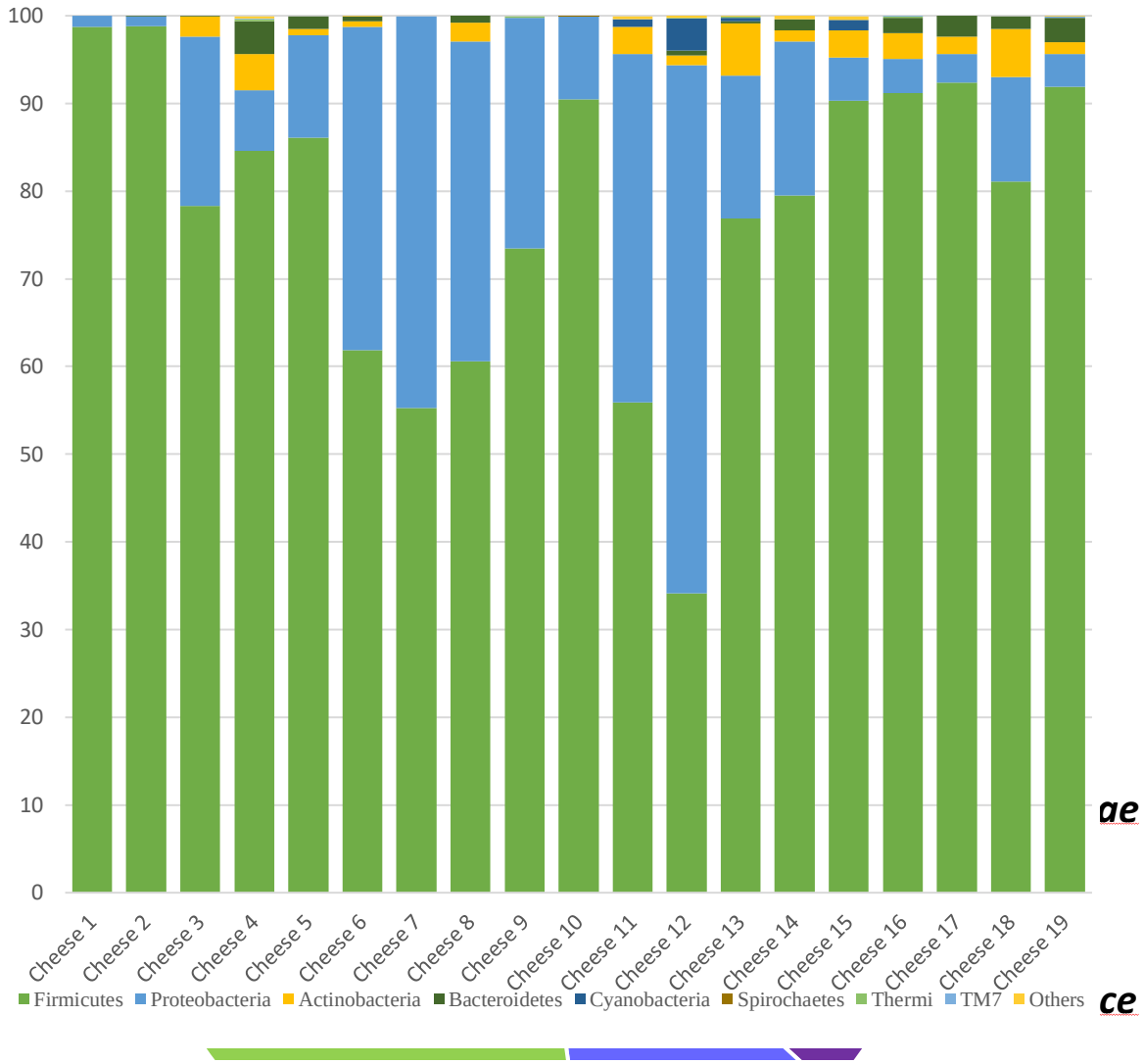



Figure 7 Relative abundance of OTUs at the Phylum level

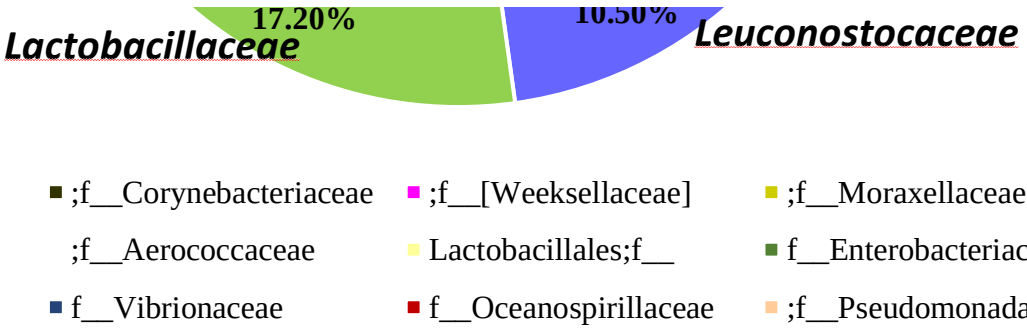



Figure 8 Relative abundance of OTUs at the family level, only OTUs with relative abundance ≥1 % in at least one sample are shown.

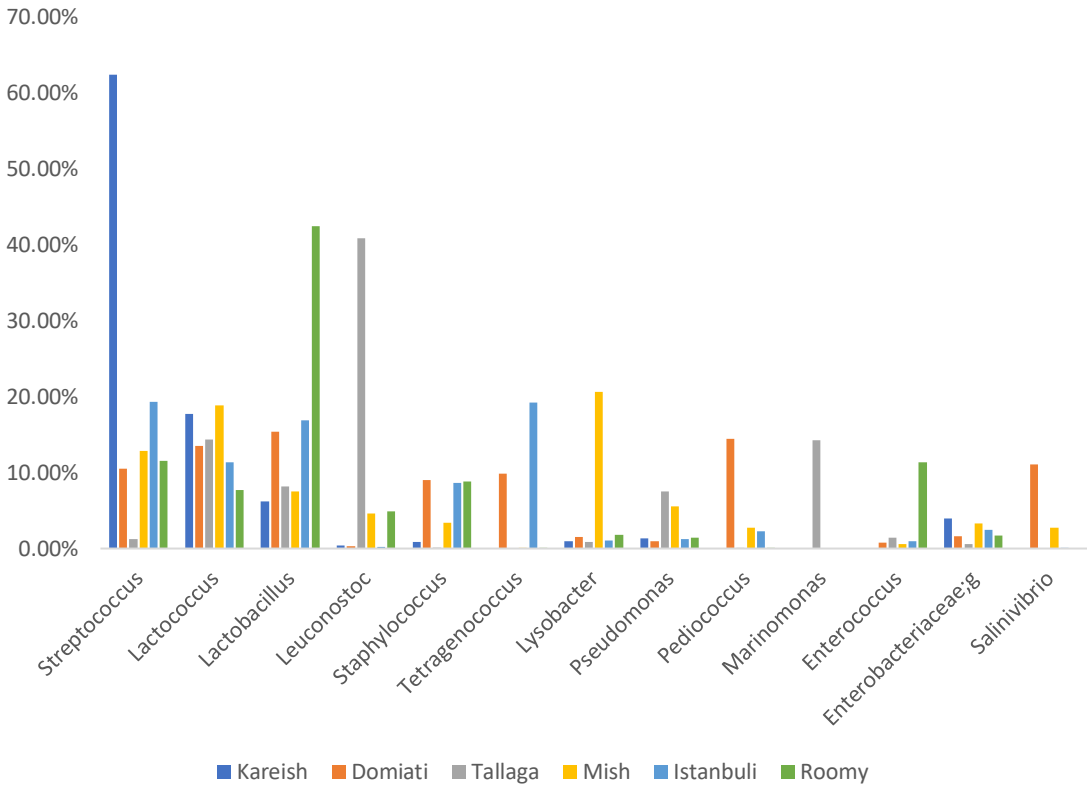



Figure 9 prevalence of dominant bacterial genera with significant differences among cheese types (Karish, Domiati, Tallaga, Mish, Istanbuli, Romy)

Values are average of genus concentration between the same cheese types.

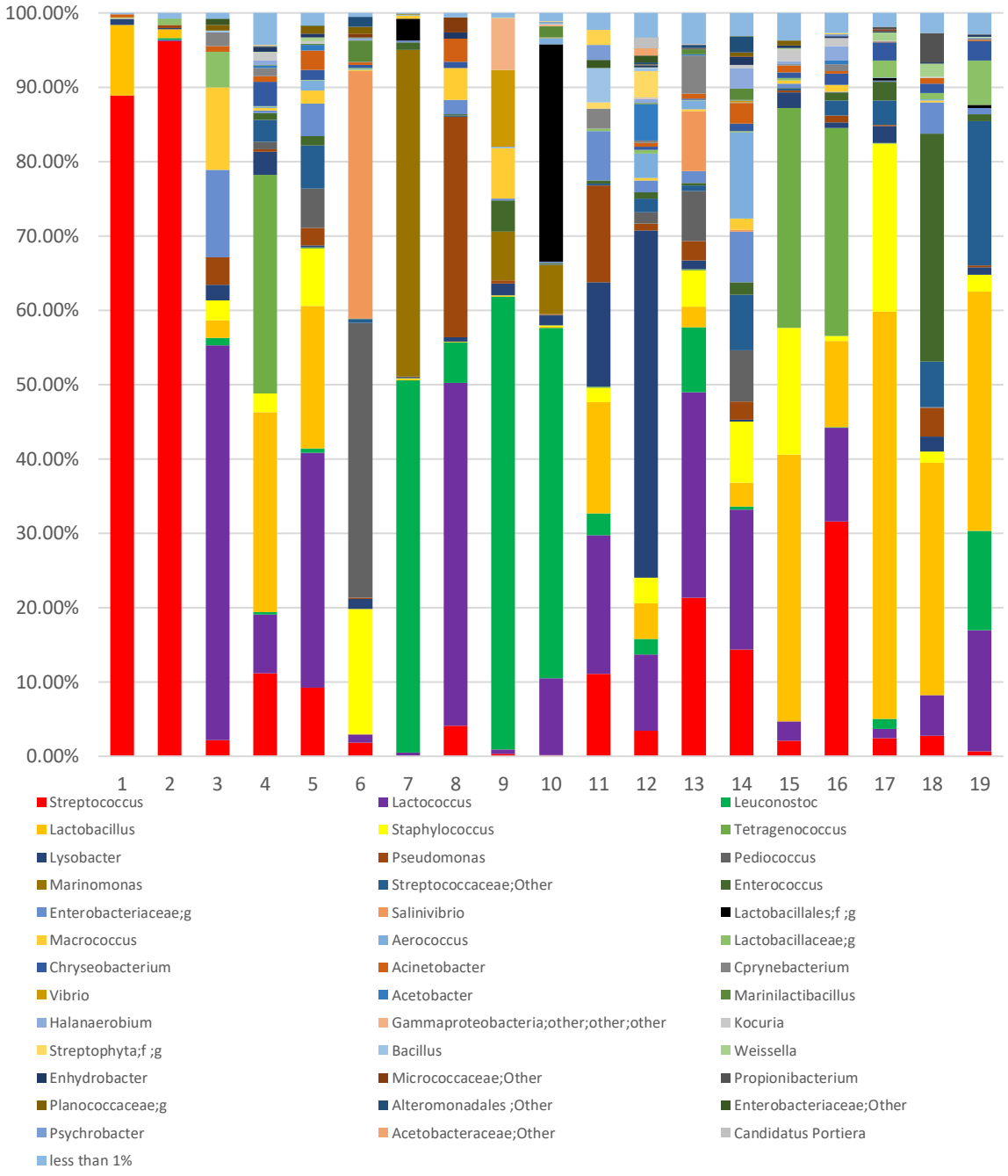



*Figure 10 Relative abundance of OTUs at the genus level considering all cheeses, only OTUs with relative abundance ≥1 % in at least one sample are shown.*

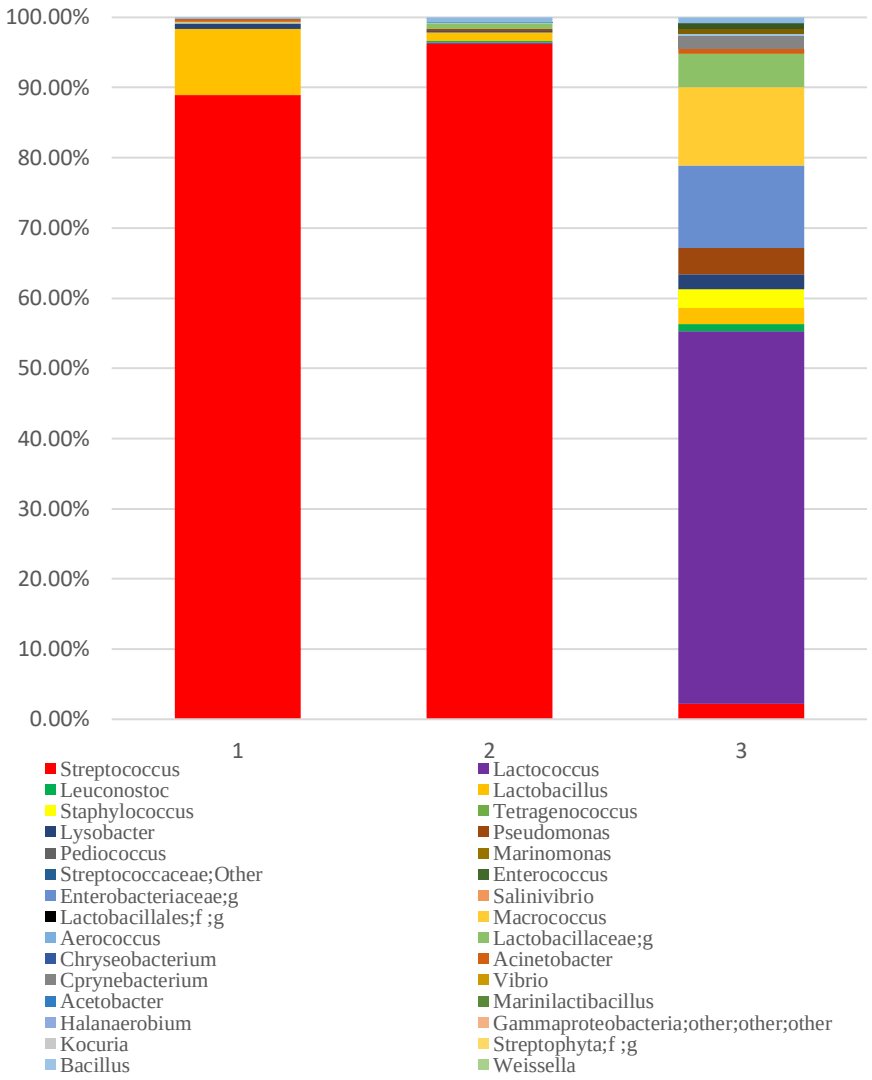



Figure 11 Relative abundance of OTUs at the genus level in Karish cheese, only OTUs with relative abundance ≥1 % in at least one sample are shown

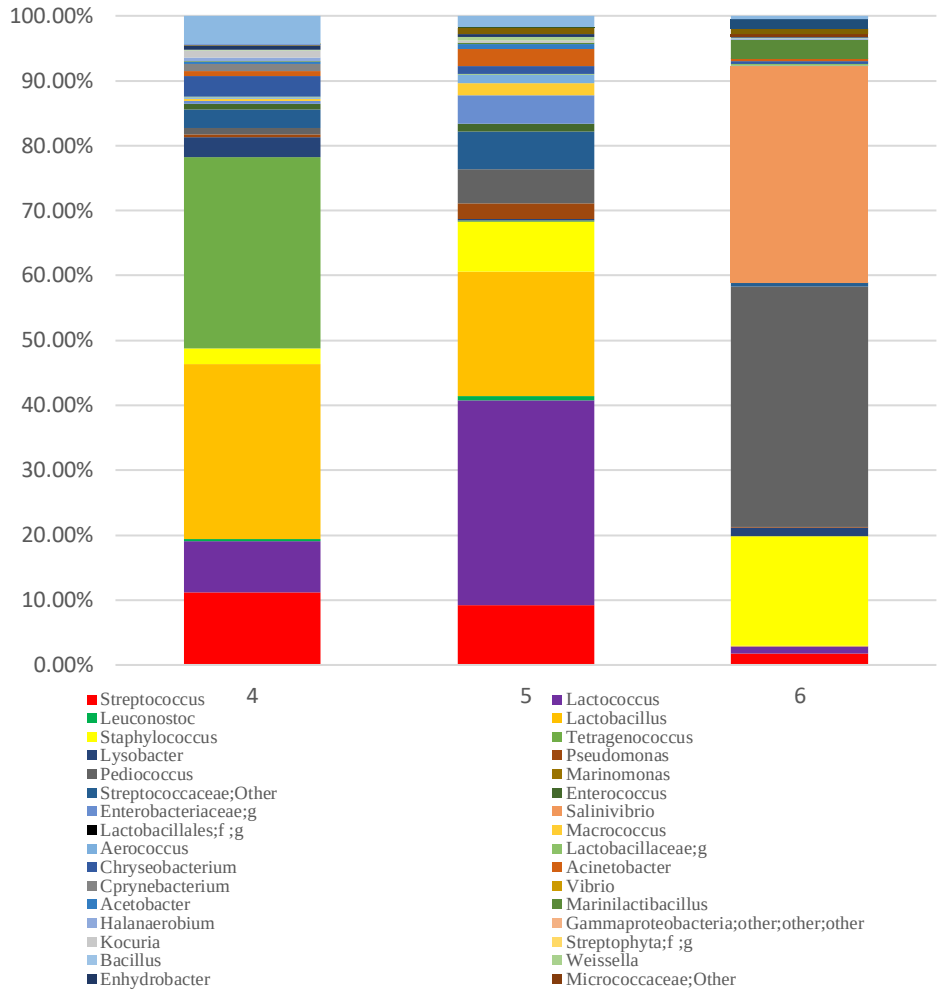



Figure 12 Relative abundance of OTUs at the genus level in Domiati Cheese, only OTUs with relative abundance ≥1 % in at least one sample are shown.

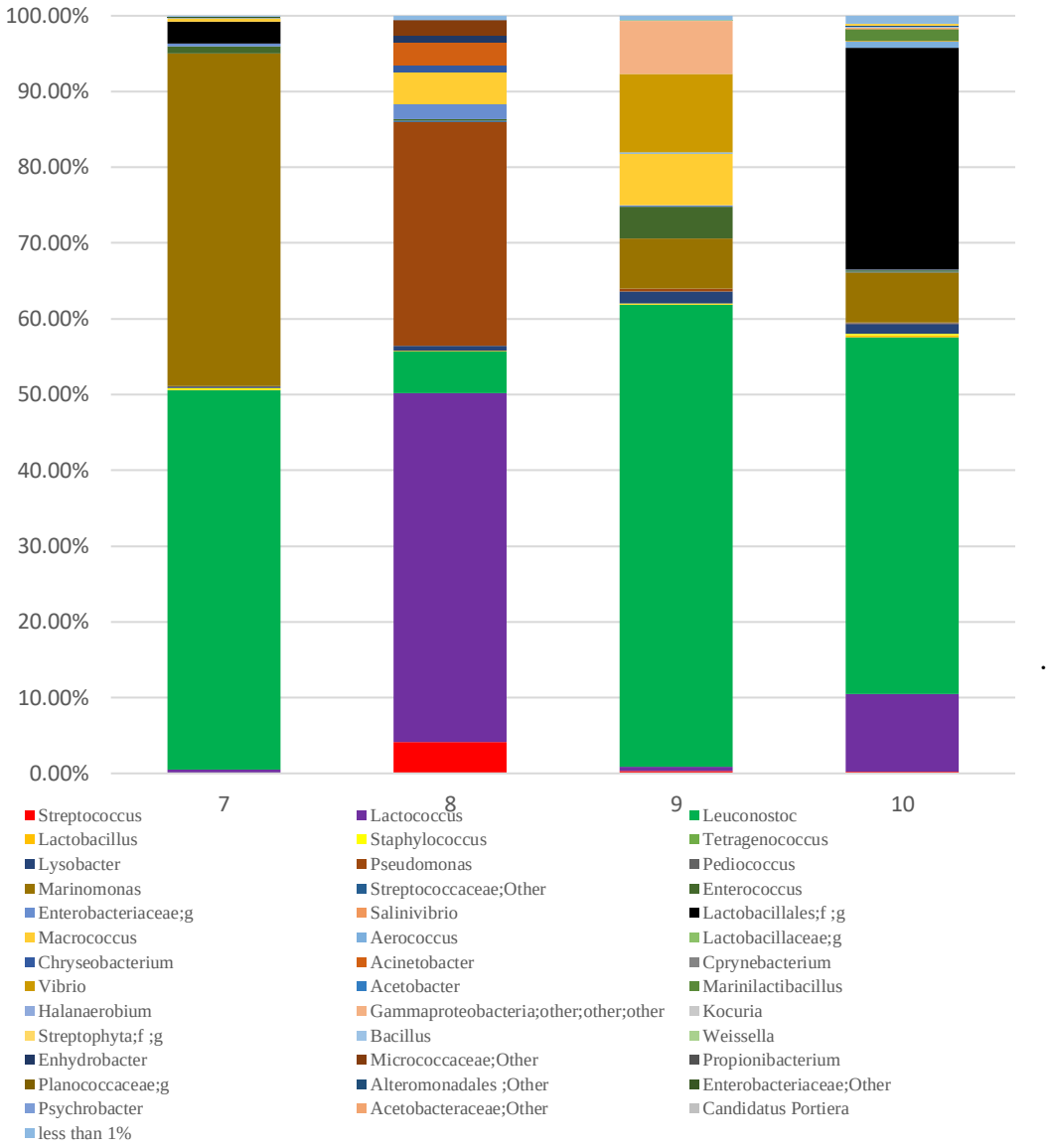



Figure 13 Relative abundance of OTUs at the genus level in Tallaga Cheese, only OTUs with relative abundance ≥1 % in at least one sample are shown.

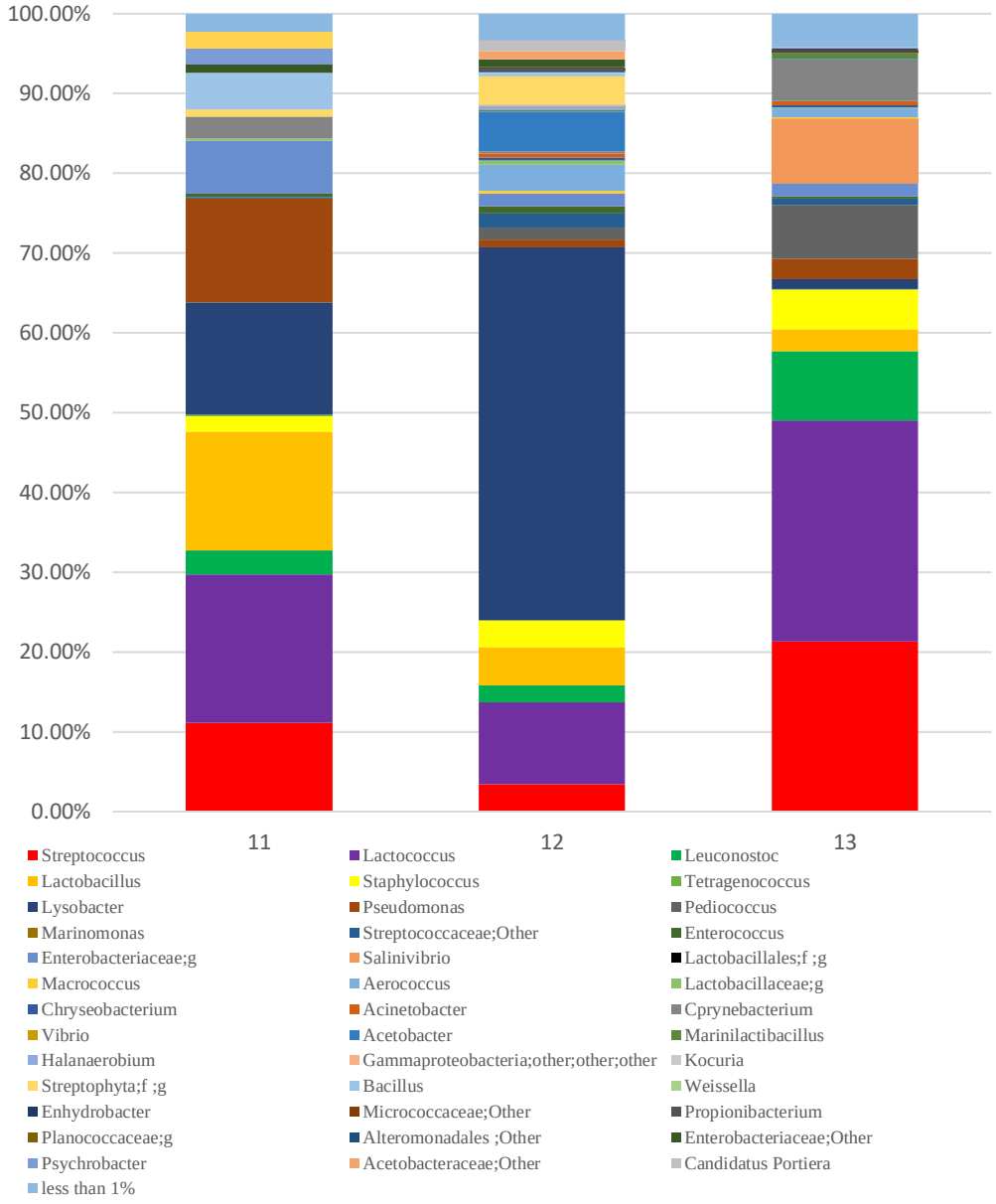



Figure 14 Relative abundance of OTUs at the genus level in Mish Cheese, only OTUs with relative abundance ≥1 % in at least one sample are shown.

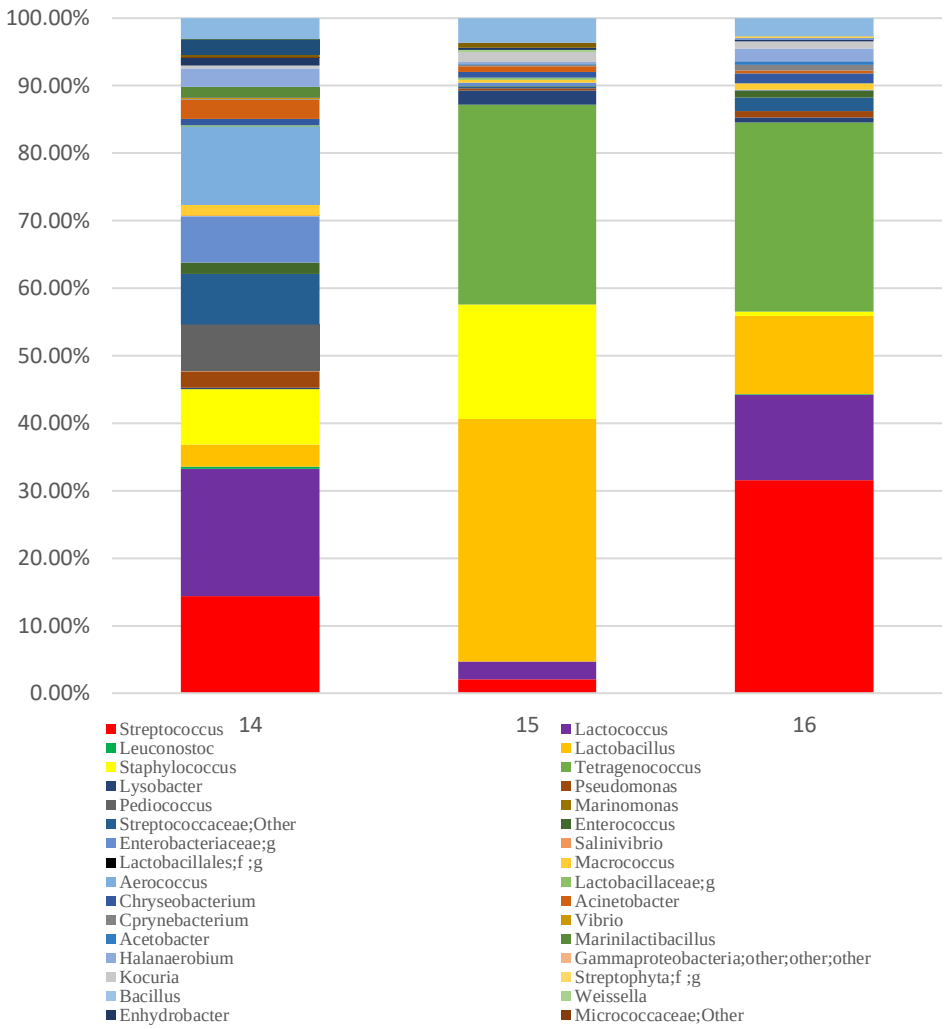



Figure 15 Relative abundance of OTUs at the genus level in Istanbuli Cheese, only OTUs with relative abundance ≥1 % in at least one sample are shown.

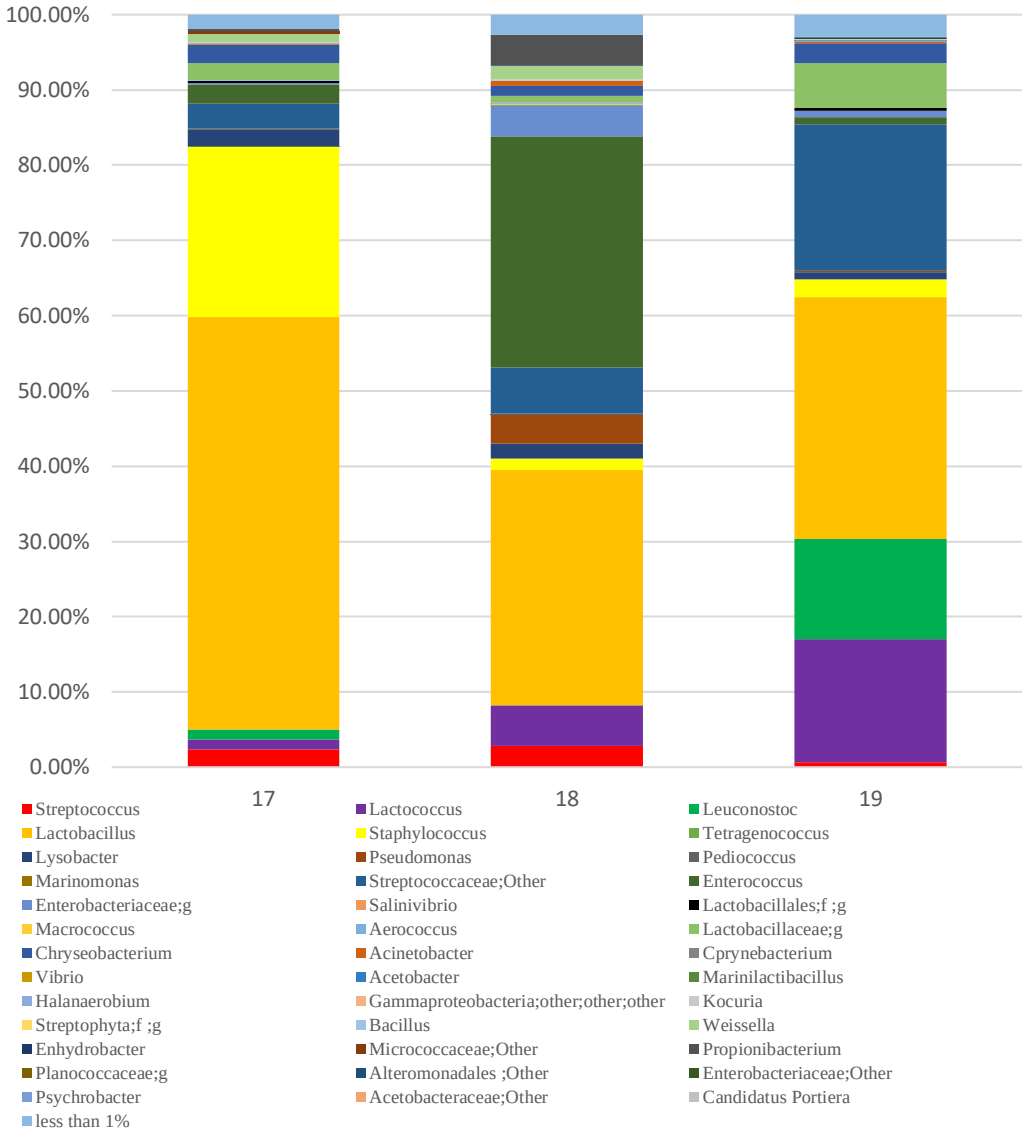



Figure 16 Relative abundance of OTUs at the genus level in Romy Cheese, only OTUs with relative abundance ≥1 % in at least one sample are shown.

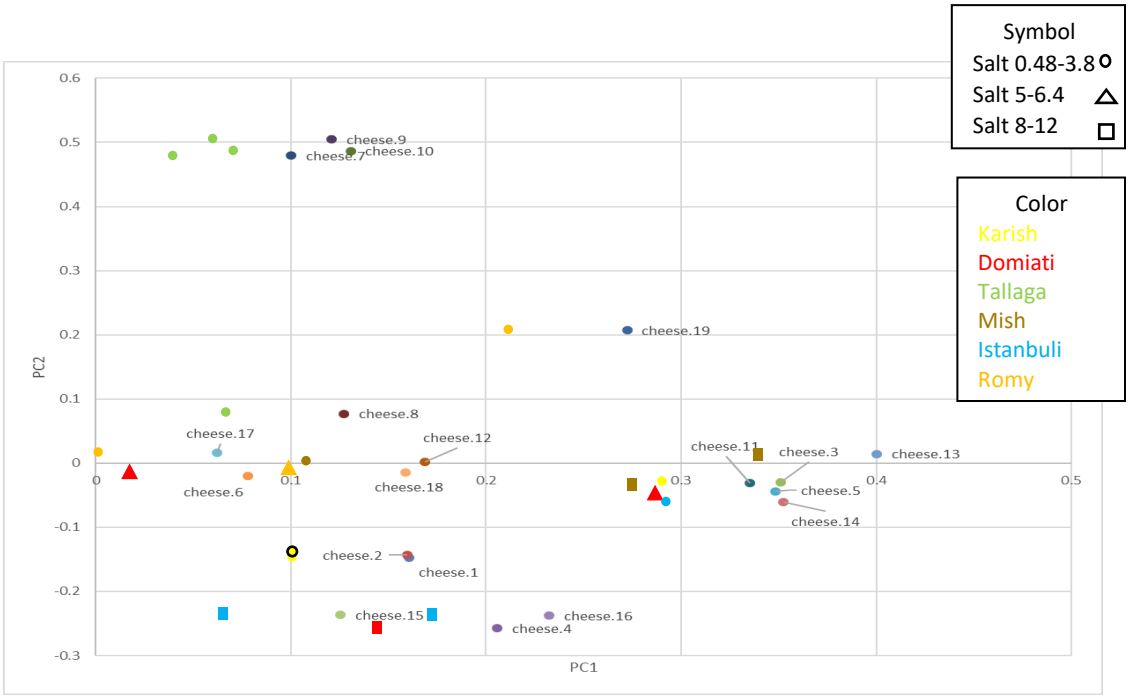



Figure 17 Principle Component Analysis (PCA) of cheese microbiota at OUT level according to cheese type and salt concentration.

# Chapter 3: Establishing an organic acid based control method for Enterobacteriaceae in traditional cheese

## Section 1: In vitro Evaluation of the ability of organic acids to control the growth of Enterobacteriaceae

### Introduction

Food preservation is a matter of increasing concern to the modern consumer in terms of safety and healthiness of the used preservative. Organic acids (OAs) are one of the common used biopreservatives in food that formed naturally in fermented products due to the microbial activity and chemical reactions. OAs are group of acidic compounds with low-molecular weight, carbohydrates in nature, that contain one or more carboxyl group -COOH in their chemical structure to which an OA group or a hydrogen atom are attached. (Anyasi et al., 2018), the number of this functional carboxyl group differ between individual OAs, for example; among the common used OAs in food, each of acetic acid and lactic acid has one carboxyl group, while malic acid and citric acid have 2 and 3 groups respectively (Theron and Lues, 2011)., the chemical structures of those OAs are shown in table 6. According to Mani-Lopez et al. (2012) the daily intake of those OAs has no limitation for human to be consumed.

The antimicrobial action of an OAs greatly affected by its pKa "acid constant"; that is the pH at which the state of equilibrium between undissociated and dissociated form of the acid exists, a higher antimicrobial action is achieved with the decrease of pH towards the pKa (Stratford and Eklund, 2003). The pKa of different OAs is shown in table 6. The mechanism of OA's action on bacterial cell is attributed to its lipophilic characteristic at the undissocaited (uncharged) form that allows the OA's penetration into the bacterial cell membrane, once inside the cytoplasm where a relatively neutral pH exists; the OA is dissociated resulting in accumulation of charged molecules and decreases in the intracellular pH resulting in impairment

of many functions (ATP, RNA, Protein synthesis) with further interruption of cellular growth (Yasothai and Giriprasad, 2015). Furthermore; the accumulation of anions inside the cell may increase the osmotic pressure with a subsequent impact on the membrane function and the metabolites availability (Hirshfield et al., 2003).

The exact action of OA in microorganisms was proposed as shown in fig. 18; in a low pH environment the undissocaited form (HA) of the acid will penetrate the microbial cell membrane, dissociate inside the cytoplasm with accumulation of charged molecules, those charged molecules will lower the internal pH, thus requires active transport to efflux protons (H+) in order to maintain the neutral pH inside the cell by getting rid of extra proton to the outside of the cytoplasm, this situation will result in consumption of ATP essential for cellular energy. After a period of time energy depletion and increasing the osmolarity will affect the cellular growth by affecting enzymes, structural proteins, and DNA functions. Malic and citric acids have also exhibited an outer membrane destabilization by chelation or intercalation (Anyasi et al., 2018).

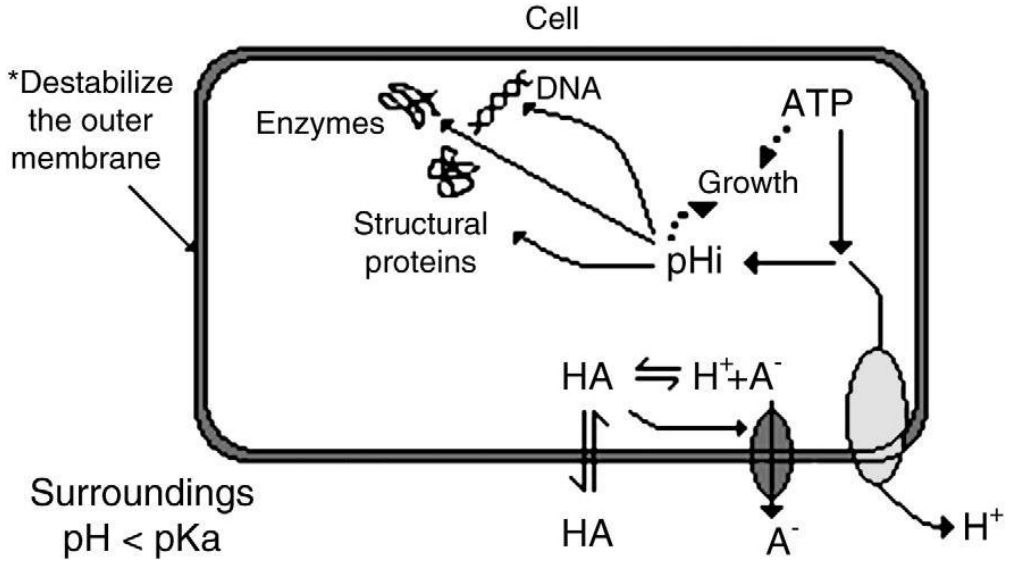


Figure 18 Mode of Action of OA on microbial cells (Mani-Lopez et al., 2012).

OAs are known to be safe (GRAS), cheap, environmentally friendly, easily manipulated and has no consumption limitation, therefore; their application as preservatives in food is commonly known, they have shown a wide antibacterial spectrum against gram positive and gram negative bacteria, among them Enterobacteriaceae including *E.coli* and *Salmonella* species have been reported before to be inhibited by the addition of different OAs like lactic acid, citric acid and acetic acid (Ben Braïek and Smaoui, 2021).

OAs can be used individually or in combinations. The application of OAs in combination has been described to exhibit a synergistic effect against food pathogens (Anyasi et al., 2018; Ben Braïek and Smaoui, 2021) for instance; It was suggested that the combination of lactic and acetic acids showed a synergistic effect with increased the action of acetic acid due to the initial lowering effect of lactic acid on pH (Helander et al., 1997), it was also Reported that the total viable count was declined to undetectable level when propionic acid was combined with either lactic or acetic acid compared to single application (Dubal et al., 2004).

The microbiological safety of the Egyptian cheese seemed to be interrupted by the significant viable count of EB investigated in chapter 1 of the current study, EB are one of the major harmful bacteria in food, it includes a wide range of bacteria that can be a source for food borne diseases or a cause of cheese defect, therefore it is necessary to prevent their growth in cheese matrix. In this chapter organic acids were investigated for their ability to inhibit different species of EB in vitro without affecting LAB important for cheese making and final properties; therefore in section 1 the Minimum Inhibitory Concentration (MIC) values of various OAs against both EB and LAB were determined, furthermore; the possible synergistic effect of their combination in sub MIC values was investigated.

## Material and Methods

### Bacterial strains

In the present work the following 8 bacterial strains belonging to EB family were used as test organisms:

① *E.coli* (ATCC 11775)

② S.Typhimurium (ATCC 13311)

③ *Acinetobacter baumannii* (JCM 6841)

④ *Raoultella planticola* (JCM 7251)

⑤ *Enterobactor aerogenes* (ATCC 43175)

⑥ *Enterobacter cloacae*(IAM 12349)

⑦ *Serratia marcescens* (IAM 12142)

⑧ *Proteus vulgaris* (IAM 12542)

All reference bacterial cultures were stored at -80° C, bacterial cultures were grown on solid agar tube media and then prepared by activation of previously cultivated strains through double transfer in TSB (BD)broth media and incubation at 37°C for 24 h.

For examined LAB; the following five strains were used

① *L. plantrum*

② *Le. mesenteroides*

③ *Le. citreum*

④ *Le. brevis*

⑤ *W. viridescens*

LAB strains were obtained from a food processing plant and stored at -80° C precultured by inoculation into 10 mL of MRS broth (Oxoid) and incubation at 30 ° C overnight then 10 µL of this preculture solution was transfered into 10 mL of fresh MRS broth (Oxoid) and incubated at 30 ° C. overnight to obtain a bacterial suspension of 9 log CFU/mL.

**Organic Acids**

The antibacterial effect of lactic acid (LA), acetic acid (AA), malic acid (MA) and citric acid (CA) (FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan) were examined against EB and LAB strains.

**MIC determination**

For determining MIC of each examined OA, Standard broth dilution method was used. 10 ml tubes of Muller-Hinton broth medium for EB and Gifu Anaerobic broth Medium "GAM" (Nissui Pharmaceutical Co., Tokyo, Japan) for LAB, supplemented with different concentrations of tested OA (0, 1250, 2500, 5000 and 10000 ppm) were prepared.

Three mL of each medium containing OA was transferred into smaller test tubes, then 0.3 mL of each of the tested bacterial solutions were inoculated into the five concentrations of each examined OA (0 ppm was used as a control). EB tubes were incubated at 37 °C, and LAB at 30° C. Tubes were visually examined after 24 and 48 h for the visible growth of tested bacteria as indicated by turbidity. MIC values were determined based on the results at 48h.

**Synergistic effect**

The result of MIC values was used to examine the possible antibacterial synergistic effect of combination of two organic acids against all tested EB (except *Proteus vulgaris*) in a concentration lower than their individual MICs. Three types of combinations (lA+CA, LA+MA and MA+CA) with different concentrations of each individual OA based on MIC value for each bacterial species were added to tubes of Muller-Hinton broth and inoculated with 5 log CFU/mL of tested organism, then all tubes were incubated at 37°C and examined after 24 and 48 h. for microbial visible growth, all examined combinations are listed in Table 7 (24 h) and 8 (48 h).

For LAB; 10000 ppm of each tested OA in six types of combinations (lA+AA, LA+MA, LA+CA, AA+MA, AA+CA and MA+CA) for all LAB strain were examined in tubes of GAM broth containing a total of 20000 ppm of OAs' combinations and inoculated with 5 log CFU/mL of tested bacteria. Tubes were incubated at 30° C and examined at 24 and 48 h as well.

**Fractional Inhibitory Concentration (FIC)**

A synergistic effect is known to be the greater effect of combining two or more substances comparing to the sum of their individual effects (Foucquier and Guedj, 2015). The possible synergistic effects of combining two organic acids against EB were expressed as FIC index, FIC index was calculated like previously described (Martin, 2010; Peh et al., 2020) as follow:

FIC of each OA was first calculated according to the following equation;

FIC of an OA = MIC of an OA in combination /MIC of an OA alone.

Then FIC Index was calculated by addition of FIC values of two OAs.

A synergistic effect was considered between a combination of two OAs only when FIC index was ≤0.5, while FIC index >0.5 and ≤1.0 was considered additive, FIC index >0.1 and ≤2.0 no difference, and FIC index > 2 was interpreted as antagonistic effect.

## Results

### MIC of Individual OAs

Lactic, Acetic, Malic and citric acids were examined for their antibacterial effect against both EB and LAB, the result was observed by visible signs of bacterial growth in their specific broth media fortified with the tested OA comparing to a control sample with no OA supplemented. Results are illustrated in Fig. 19 (EB) and 20 (LAB) after 24 (A) and 48 (B) h.; the darker the color in the figure the more turbidity in the media was indicated as a result of the extent of bacterial growth, white color indicates clear appearance of the broth as a result of complete inhibition of the bacterial growth.

MIC values were determined based on the complete inhibition of the bacteria (indicated by white color) after 48 h, even if some bacterial growth was observed with the same OA concentration at 24 h. MIC values of the four OAs against individual EB species is shown in Table 7. Generally all tested species of EB were inhibited by at least one OA within the examined concentrations with different MIC values. Acetic acid showed the highest inhibitory effect represented by the lowest MIC values (1250 ppm) for all EB except *Proteus vulgaris* (MIC = 2500 ppm) that was affected more by malic and citric acids with an MIC value of 1250

each. The second strongest OA was lactic acid that recorded an MIC value of 2500 ppm against all tested EB except *Enterobacter aerogenes* that was not inhibited up to 10000 ppm of LA and *S. Typhimurium* that required an MIC of 5000 ppm of LA, while malic and citric acid had the lowest antibacterial effect within the tested OAs against EB, both *Enterobacter aerogenes* and *Serratia marcescens* were not inhibited by 10000 ppm of MA or CA, furthermore MA was not able to inhibit *Enterobacter cloacae*by 10000 ppm, and 5000 ppm MIC of MA required to inhibit *Raoultella planticola*, on the other hand MA showed its strongest antibacterial effect against *S. Typhimurium* (MIC =1250 ppm), and both *E. coli* and *Acinetobacter baumannii* was totally inhibited by 2500 ppm of MA. The lowest MIC for CA was observed for both *Acinetobacter baumannii* and *Proteus vulgaris* (1250 ppm), followed by 2500 ppm of MIC for both *E. coli* and *Raoultella planticola* and 5000 ppm for *S. Typhimurium* and *Enterobacter cloacae*.

As for LAB, all examined tubes appeared with high turbidity similar to that of the control sample (Figure 21) after 24 and 48 h of incubation at 30°C, which indicates that examined OAs have no inhibitory effect against tested LAB in the current study.

**Synergistic effect**

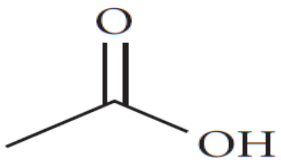
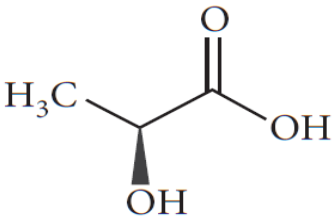
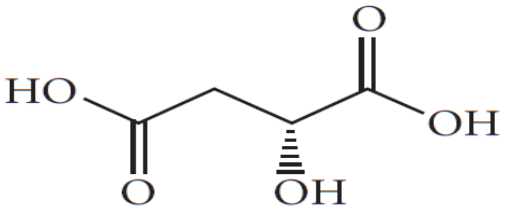
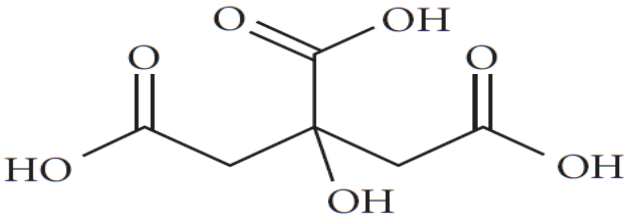
Three OAs (lactic, citric and malic acids) were examined for their possible double combinations (lA+CA, LA+MA and MA+CA) with different concentrations lower than their individual MIC against 7 species from EB group, results after 24h is shown in Table 7 and after 48 h is shown in Table 8 . The observed bacterial growth in broth media indicated that *E.coli* was not inhibited by half MIC (1250 ppm) of LA and CA together after 24 and 48 h, while the same combination showed a growth inhibition for *S. Typhimurium* after 48 h (which is considered 0.25 MIC for this bacteria) and after 24 and 48 h for *Raoultella planticola*, *Enterobacter a* and *Serratia marcescens*. When the concentration of CA increased to 0.5 MIC (2500 ppm) with 1250 ppm LA inhibition of the growth of *S. Typhimurium* was observed from the first day, while a further increase of LA concentration to 2500 ppm combined with 2500 pm of CA showed no inhibitory effect for the same bacteria at all. For Acinetobacter baumannii; a combination of half MIC from lactic and malic acids (1250 ppm each) showed no inhibitory effect after 1 and 2 days.

All five combinations tested against *Raoultella planticola* exhibited an antibacterial effect at the two point observations (24 and 48 h). *Enterobacter aerogenes's* growth was inhibited by 3 out of 12 tested combinations after 24 and 48 h (LA 1250+CA 1250, LA 2500+CA 1250, and MA 1250+ CA 2500). Eight combinations were examined against *Enterobacter cloacae*; of them MA 1250 +CA 2500 showed a growth inhibition only after 24 h but not 48h, while the opposite was observed for combining greater concentration of MA 2500 and lower of CA 1250 where the bacteria grown at 24 h but growth inhibition was observed after 48 h. furthermore the growth inhibition of *Serratia marcescens* was achieved after 24 h with 13 out of 14 different combinations, and the growth inhibition after 48 h decreased to 12 combinations, for this bacteria different results after 24 and 48 h was observed for 3 combinations (MA 1250+CA 1250, MA 1250+CA 2500 and MA 5000+CA 2500).

Regarding LAB; a sum of 20000 ppm of two combined OAs (6 combinations) had no inhibitory effect against any of the five tested bacteria after 24 and 48 h.

The observed antibacterial effect of OAs combinations after 48 h was used for calculating FICs Index, the resultant inhibition of 24 combiantions against 5 EB with their FIC index values are shown in Table 9. Results of FIC calculation indicated that 4 combinations had FIC values of 0.5 or less that was considered as "synergistic effect", they included LA 1250 ppm + CA 1250 ppm against both *S. Typhimurium* and *Enterobacter aerogenes* and MA 2500 ppm + CA 1250 ppm against both *Enterobacter cloacae*and *Serratia marcescens*, while the other 20 combinations showed FIC index >0.5 and ≤1.0 and were interpreted as additives.

**Table 6. Chemical structure of common organic acids.**

| **Organic acid** | **Molecular formula** | **Structural formula** | pKa |
|---|---|---|---|
| Acetic acid | $C_2H_4O_2$ |  | 4.76 |
| Lactic acid | $C_3H_6O_3$ |  | 3.9 |
| Malic acid | $C_4H_6O_5$ |  | 3.46; 5.10 |
| Citric acid | $C_6H_8O_7$ |  | 3.13; 4.76; 6.4 |

Source: Ben Braïek and Smaoui (2021)

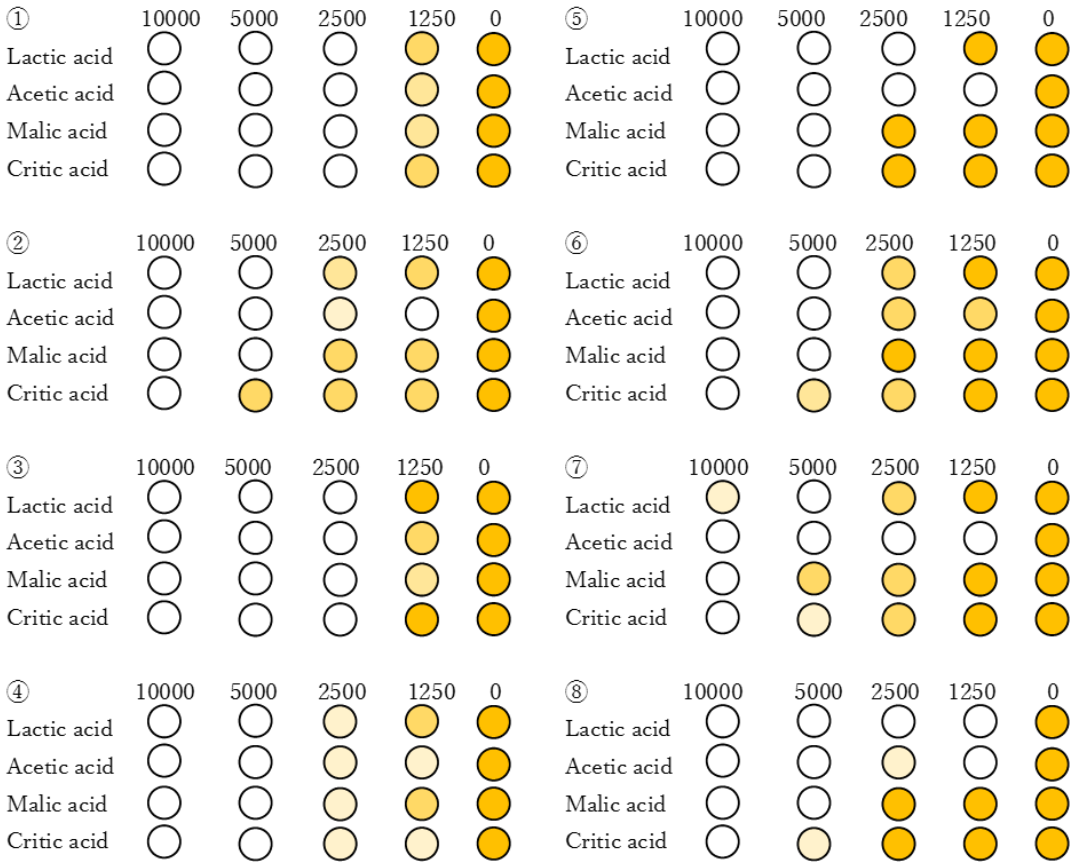



**Figure 19 (A) In vitro visible growth inhibition of EB group after 24 h using OAs (**0,1250,2500,5000 and 10000 ppm**),** ① *E.coli* , ② *S.Typhimurium* ,③*Acinetobacter baumannii* ④*Raoultella planticola* , ⑤*En*

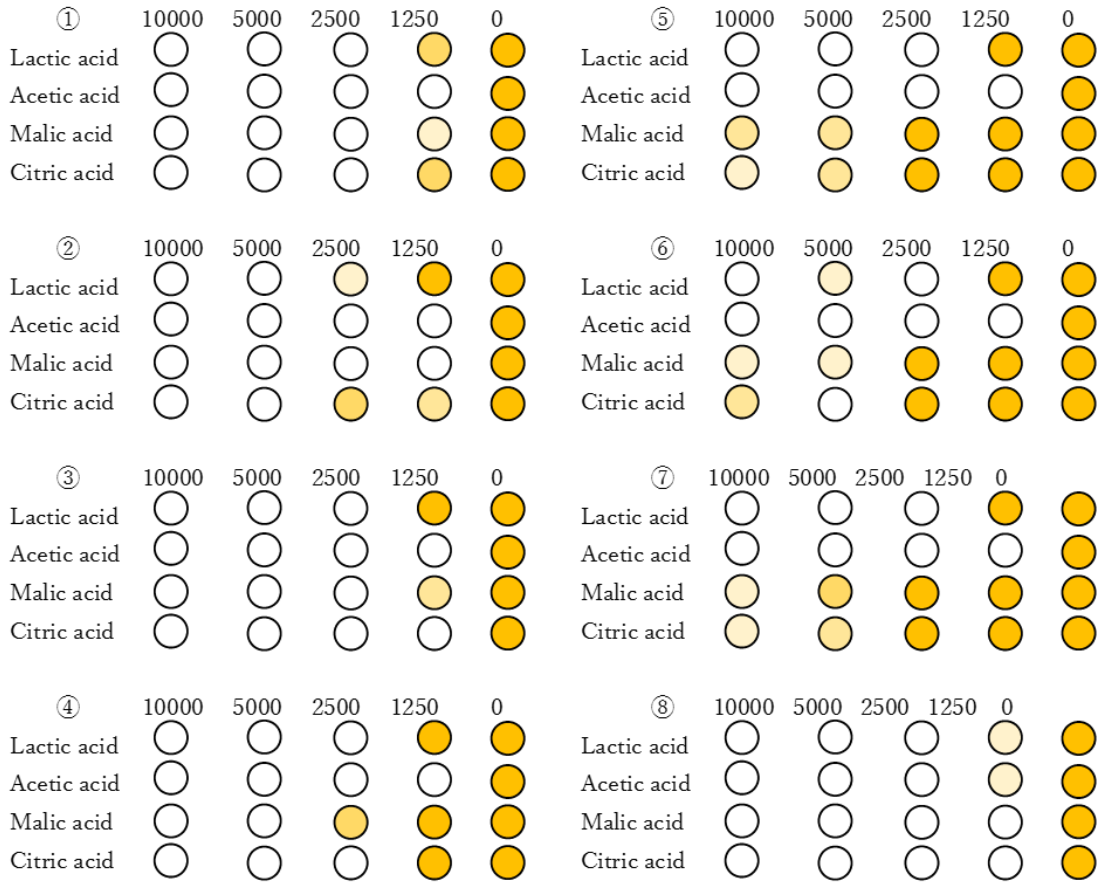



**Figure 20 (B) In vitro visible growth inhibition of EB group after 48 h using OAs (**0,1250,2500,5000 and 10000 ppm**),** ① *E.coli* , ② *S.Typhimurium* ,③*Acinetobacter baumannii* ④*Raoultella planticola* , ⑤*Enterobactor aerogenes*,⑥*Enterobacterr cloacae* , ⑦*Serratia marcescens* ,⑧*Proteus vulgaris*.

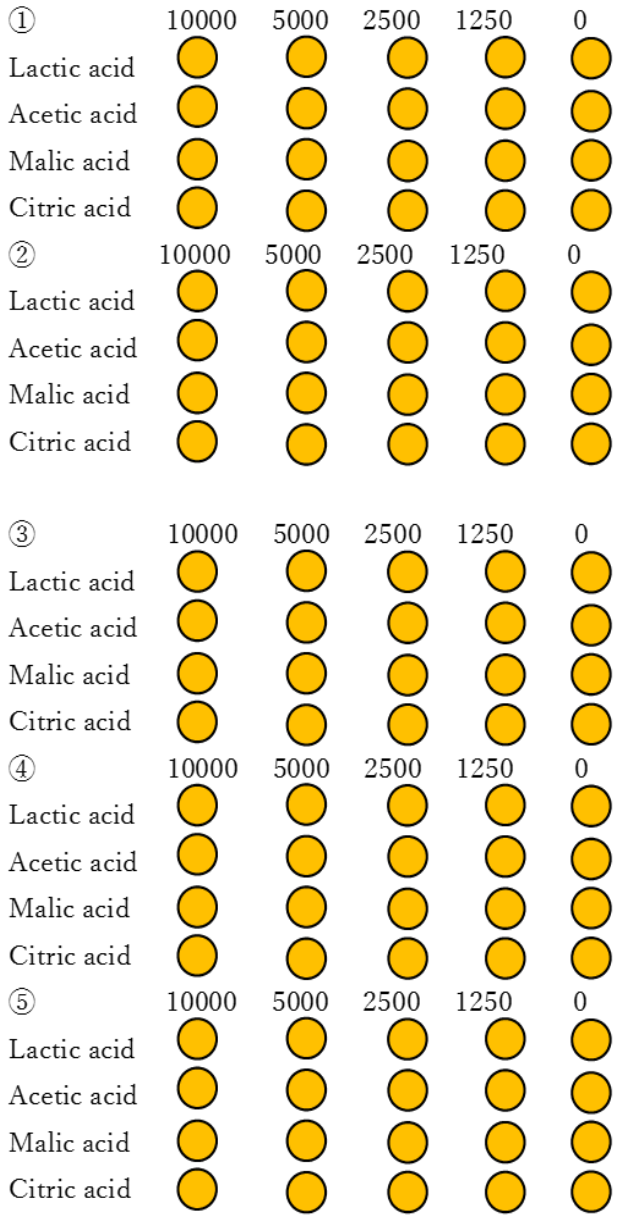



**Figure 21 (A) In vitro visible growth inhibition of LAB after 24 h using OAs (**0, 1250, 2500,5000 and 10000 ppm**),** ①*L. plantrum*, ②*Le. Mesenteroides*, ③*Le. Citreum*, ④*Le.Brevis*,⑤ *W. viridescens*.

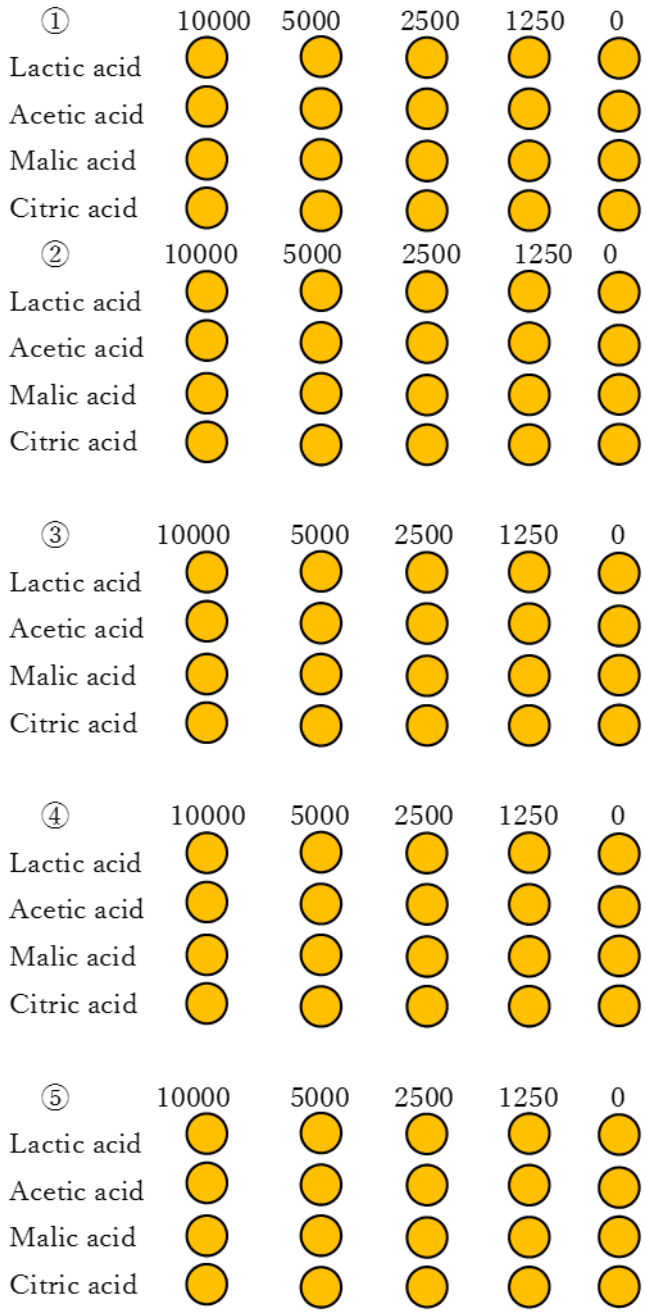



**Figure 22 (B) In vitro visible growth inhibition of LAB after 48 h using OAs (**0, 1250, 2500,5000 and 10000 ppm**),** ①*L. plantrum*, ②*Le. Mesenteroides*, ③*Le. Citreum*, ④*Le.Brevis*,⑤ *W. viridescens*.

**Table 7. MIC values of tested organic acids against EB**

| Tested Bacteria | MIC (ppm) | | | |
|---|---|---|---|---|
| | **Lactic** | **Acetic** | **Malic** | **Citric** |
| ① *E. coli* | 2500 | 1250 | 2500 | 2500 |
| ② *S. Typhimurium* | 5000 | 1250 | 1250 | 5000 |
| ③ *Acinetobacter baumannii* | 2500 | 1250 | 2500 | 1250 |
| ④ *Raoultella planticola* | 2500 | 1250 | 5000 | 2500 |
| ⑤ *Enterobacter aerogenes* | >10000 | 1250 | >10000 | >10000 |
| ⑥ *Enterobacterr cloacae* | 2500 | 1250 | >10000 | 5000 |
| ⑦ *Serratia marcescens* | 2500 | 1250 | >10000 | >10000 |
| ⑧ *Proteus vulgaris* | 2500 | 2500 | 1250 | 1250 |

**Table 8.** Combinations of organic acids and their antibacterial effects against EB after 24 h.

| TESTED EB | COMBINATIONS | | VISIBLE INHIBITION EFFECT |
|---|---|---|---|
| ①*E. COLI* | Lactic acid 1250 | Citric acid 1250 | × |
| ②*S. TYPHIMRIUM* | Lactic Acid 1250 | Citric acid 1250 | × |
| | Lactic Acid 1250 | Citric acid 2500 | ○ |
| | Lactic Acid 2500 | Citric acid 2500 | × |
| ③*ACINETOBACTER BAUMANNII* | Lactic Acid 1250 | Malic acid 1250 | × |
| ④*RAOULTELLA PLANTICOLA* | Lactic Acid 1250 | Malic acid 1250 | ○ |
| | Lactic Acid 1250 | Malic acid 2500 | ○ |
| | Lactic Acid 1250 | Citric acid 1250 | ○ |
| | Malic acid 1250 | Citric acid 1250 | ○ |
| | Malic acid 2500 | Citric acid 1250 | ○ |
| 5 *ENTEROBACTER AEROGENES* | Lactic Acid 1250 | Malic acid 1250 | × |
| | Lactic Acid 1250 | Malic acid 2500 | × |
| | Lactic Acid 1250 | Citric acid 1250 | ○ |
| | Lactic Acid 1250 | Citric acid 2500 | × |
| | Lactic Acid 2500 | Malic acid 1250 | × |
| | Lactic Acid 2500 | Malic acid 2500 | × |
| | Lactic Acid 2500 | Citric acid 1250 | ○ |
| | Lactic Acid 2500 | Citric acid 2500 | × |
| | Malic acid 1250 | Citric acid 1250 | × |
| | Malic acid 1250 | Citric acid 2500 | ○ |
| | Malic acid 2500 | Citric acid 1250 | × |
| | Malic acid 2500 | Citric acid 2500 | × |
| 6 *ENTEROBACTER CLOACAE* | Lactic Acid 1250 | Malic acid 1250 | × |
| | Lactic Acid 1250 | Malic acid 2500 | × |
| | Lactic Acid 1250 | Citric acid 1250 | × |
| | Lactic Acid 1250 | Citric acid 2500 | × |
| | Malic acid 1250 | Citric acid 1250 | × |
| | Malic acid 1250 | Citric acid 2500 | ○ |
| | Malic acid 2500 | Citric acid 1250 | × |
| | Malic acid 2500 | Citric acid 2500 | × |
| 7 *SERRATIA MARCESCENS* | Lactic Acid 1250 | Malic acid 1250 | ○ |
| | Lactic Acid 1250 | Malic acid 2500 | ○ |
| | Lactic Acid 1250 | Malic acid 5000 | ○ |

| | | |
|---|---|---|
| Lactic Acid 1250 | Citric acid 1250 | ○ |
| Lactic Acid 1250 | Citric acid 2500 | ○ |
| Lactic Acid 1250 | Citric acid 2500 | ○ |
| Malic acid 1250 | Citric acid 1250 | ○ |
| Malic acid 1250 | Citric acid 2500 | ○ |
| Malic acid 1250 | Citric acid 5000 | ○ |
| Malic acid 2500 | Citric acid 1250 | ○ |
| Malic acid 2500 | Citric acid 2500 | ○ |
| Malic acid 2500 | Citric acid 5000 | ○ |
| Malic acid 5000 | Citric acid 1250 | ○ |
| Malic acid 5000 | Citric acid 2500 | × |
| Malic acid 5000 | Citric acid 5000 | ○ |

**Table 9.** Combinations of organic acids and their antibacterial effects against EB after 48 h

| TESTED EB SPECIES | COMBINATION | | VISIBLE EFFECT | INHIBITION |
|---|---|---|---|---|
| ①*E. COLI* | Lactic Acid 1250 | Citric acid 1250 | × | |
| ②*S. TYPHIMRIUM* | Lactic Acid 1250 | Citric acid 1250 | ○ | |
| | Lactic Acid 1250 | Citric acid 2500 | ○ | |
| | Lactic Acid 2500 | Citric acid 2500 | × | |
| ③*ACINETOBACTER BAUMANNII* | Lactic Acid 1250 | Malic acid 1250 | × | |
| ④*RAOULTELLA PLANTICOLA* | Lactic Acid 1250 | Malic acid 1250 | ○ | |
| | Lactic Acid 1250 | Malic acid 2500 | ○ | |
| | Lactic Acid 1250 | Citric acid 1250 | ○ | |
| | Malic acid 1250 | Citric acid 1250 | ○ | |
| | Malic acid 2500 | Citric acid 1250 | ○ | |
| 5 *ENTEROBACTER AEROGENES* | Lactic Acid 1250 | Malic acid 1250 | × | |
| | Lactic Acid 1250 | Malic acid 2500 | × | |
| | Lactic Acid 1250 | Citric acid 1250 | ○ | |
| | Lactic Acid 1250 | Citric acid 2500 | × | |
| | Lactic Acid 2500 | Malic acid 1250 | × | |
| | Lactic Acid 2500 | Malic acid 2500 | × | |
| | Lactic Acid 2500 | Citric acid 1250 | ○ | |
| | Lactic Acid 2500 | Citric acid 2500 | × | |
| | Malic acid 1250 | Citric acid 1250 | × | |
| | Malic acid 1250 | Citric acid 2500 | ○ | |
| | Malic acid 2500 | Citric acid 1250 | × | |
| | Malic acid 2500 | Citric acid 2500 | × | |
| 6 *ENTEROBACTER CLOACAE* | Lactic Acid 1250 | Malic acid 1250 | × | |
| | Lactic Acid 1250 | Malic acid 2500 | × | |
| | Lactic Acid 1250 | Citric acid 1250 | × | |
| | Lactic Acid 1250 | Citric acid 2500 | × | |
| | Malic acid 1250 | Citric acid 1250 | × | |
| | Malic acid 1250 | Citric acid 2500 | × | |
| | Malic acid 2500 | Citric acid 1250 | ○ | |
| | Malic acid 2500 | Citric acid 2500 | × | |
| 7 *SERRATIA MARCESCENS* | Lactic Acid 1250 | Malic acid 1250 | ○ | |
| | Lactic Acid 1250 | Malic acid 2500 | ○ | |
| | Lactic Acid 1250 | Malic acid 5000 | ○ | |
| | Lactic Acid 1250 | Citric acid 1250 | ○ | |
| | Lactic Acid 1250 | Citric acid 2500 | ○ | |
| | Lactic Acid 1250 | Citric acid 2500 | ○ | |
| | Malic acid 1250 | Citric acid 1250 | × | |
| | Malic acid 1250 | Citric acid 2500 | × | |
| | Malic acid 1250 | Citric acid 5000 | ○ | |
| | Malic acid 2500 | Citric acid 1250 | ○ | |
| | Malic acid 2500 | Citric acid 2500 | ○ | |
| | Malic acid 2500 | Citric acid 5000 | ○ | |
| | Malic acid 5000 | Citric acid 1250 | ○ | |

| | | |
|---|---|---|
| Malic acid 5000 | Citric acid 2500 | ○ |
| Malic acid 5000 | Citric acid 5000 | ○ |

**TABLE 10.TABLE 10. FIC INDEX OF ORGANIC ACIDS COMBINATIONS FOR INHIBITING EB.**

| EB SPECIES | combination | | FIC value | interpretation |
|---|---|---|---|---|
| ***S. TYPHIMRIUM*** | Lactic Acid 1250 | Citric acid 1250 | 0.5 | synergistic |
| | Lactic Acid 1250 | Citric acid 2500 | 0.75 | additive |
| ***RAOULTELLA PLANTICOLA*** | Lactic Acid 1250 | Malic acid 1250 | 0.75 | additive |
| | Lactic Acid 1250 | Malic acid 2500 | 1.0 | additive |
| | Lactic Acid 1250 | Citric acid 1250 | 1.0 | additive |
| | Malic acid 1250 | Citric acid 1250 | 0.75 | additive |
| | Malic acid 2500 | Citric acid 1250 | 1.0 | additive |
| ***ENTEROBACTER AEROGENES*** | Lactic Acid 1250 | Citric acid 1250 | 0.25 | synergistic |
| | Lactic Acid 2500 | Citric acid 1250 | 0.375 | additive |
| | Malic acid 1250 | Citric acid 2500 | 0.375 | additive |
| ***ENTEROBACTER CLOACAE SERRATIA MARCESCENS*** | Malic acid 2500 | Citric acid 1250 | 0.5 | synergistic |
| | Lactic Acid 1250 | Malic acid 1250 | 0.625 | additive |
| | Lactic Acid 1250 | Malic acid 2500 | 0.75 | additive |
| | Lactic Acid 1250 | Malic acid 5000 | 1.0 | additive |
| | Lactic Acid 1250 | Citric acid 1250 | 0.625 | additive |
| | Lactic Acid 1250 | Citric acid 2500 | 0.75 | additive |
| | Lactic Acid 1250 | Citric acid 2500 | 1.0 | additive |
| | Malic acid 1250 | Citric acid 5000 | 0.625 | additive |
| | Malic acid 2500 | Citric acid 1250 | 0.375 | synergistic |
| | Malic acid 2500 | Citric acid 2500 | 0.5 | additive |
| | Malic acid 2500 | Citric acid 5000 | 0.75 | additive |
| | Malic acid 5000 | Citric acid 1250 | 0.625 | additive |
| | Malic acid 5000 | Citric acid 2500 | 0.75 | additive |
| | Malic acid 5000 | Citric acid 5000 | 1.0 | additive |

## Section 2: Controlling the growth of Enterobacteriaceae in cheese matrix

## Introduction

A vast diversity of preservation methods have been employed to prevent the growth of pathogenic and spoilage microorganisms as well as extending the cheese shelf life. Refrigeration, high hydrostatic pressure processing, packaging techniques and food additives are the common intervention that have been used. Food additives especially those derived from natural sources are effective substitution for unhealthy preservatives. Studies on their application in cheese are continually conducted to improve their effectiveness and ensure sustainability (Nájera et al., 2021; Ritota and Manzi, 2020).

A long history of using OAs as food additives and preservatives against potential food contaminants owing to their ability to exhibit a bacteriostatic or bactericidal effect is well documented (Ricke, 2003). OAs are safe antimicrobial interventions that have obtained an approval of application in meat in the United States. The European union has approved the use of some OAs for specific types of cheese such as sorbic acid for unripened and ripened cheese, and acetic and lactic acid for mozzarella cheese (Ritota and Manzi, 2020).

The extend of antibacterial action of an OA is usually dependent on dual factors, one factor is related to the target microorganism and the other is related to the surrounding environment (Ricke, 2003)

The in vitro control of microorganism is needed to confirm the capability of the used antimicrobial intervention against the target bacteria in addition to determining the minimal concentration needed for growth inhibition, however once in the food matrix, an antimicrobial agent will have to overcome lots of challenges to prove its real effectiveness as a food preservative. Such challenges include factors related to the food itself; fat and protein contents of the cheese were reported to contradict the action of preservatives (Smith-Palmer et al. 2001; Gutierrez et al. 2008). Hence a higher concentration of the OA than that used in the laboratory media is usually required to show the same effect in the complex food matrix (Moro et al., 2013). Furthermore food buffering capacity and pH are among factors influencing the action of OAs.

Selecting the proper antimicrobial for cheese preservation particularly needs a careful assessment, since the antibacterial effect that may reach the natural cheese microbiota or the

added starter culture should be evaluated in parallel with the target microorganisms (Gouvea et al., 2017; De Souza et al. 2016). It is therefore important that the cheese preservative doesn't have an inhibitory effect on the growth of LAB that is necessary for cheese making and organoleptic characteristics.

OAs have shown a considerable antibacterial activity against wide range of pathogenic bacteria including different species of Enterobacteriaceae (Jamilah et al., 2008), they are extensively used in meat preservation (Ben Braïek and Smaoui, 2021), poultry (Mani-Lopez et al., 2012) and for decontamination of fresh products (Lianou et al., 2012). However their application in cheese material is scarcely studied. Therefore this study aimed to evaluate the ability of OAs individually and in combination to control the growth of EB in soft white Tallaga cheese during refrigerated storage.

## Material and Methods

### Bacterial strains

For testing the antibacterial effect of OAs in cheese, four EB reference strains were used (*E.coli* ATCC 11775, *S. Typhimurium* ATCC 13311, *Enterobacter cloacae* IAM 12349 and *Serratia marcescens* IAM 12142), the frozen strains (-80° C) were recovered by growing on Tripticase Soy Agar "TSA" (BD) agar screw-caped tubes, then inoculated into 10 mL TSB (BD), incubated at 37° C overnight to obtain a preculture solution, then 10 µL of the preculture solution was inoculated into 10 mL of newly fresh TSB and incubated at 37° C overnight to obtain a bacterial solution of about 9 log CFU/mL.

The bacterial inoculum concentration was measured by adjusting the solution to an optical density of 1 at 600 nm (OD600) using spectrophotometer to obtain a final concentration of 6 log CFU/mL to be inoculated into cheese.

### Organic Acids

Four OAs were examined against tested strains; AA, LA, CA, and MA (FUJIFILIM), in the following conditions:

AA 5000ppm

LA 10000ppm

LA 5000ppm+CA 5000ppm

MA 10000ppm+CA 5000ppm

**Cheese making**

Egyptian Tallaga cheese was chosen as a model of fresh soft cheese to evaluate the inhibitory action of tested OAs against different EB strains, cheese was manufactured using High Temperature Short Time (HTST) pasteurized cow milk previously treated at 66° C for 30 min that contained 4% fat according to the method described by El-Kholy et. al (2016) with some modifications (fig. 21), cow milk was heated to 37° C and was equally divided into 5 groups for each of the four tested EB strain, to each group microbial rennet (SIGMA-Aldrich, Louis, USA) from mucor miehi type II, sodium chloride and calcium chloride were added in a percentage of 0.05%, 0.3% and 0.02% respectively, then group 1 was inoculated with 6 log CFU/mL of one of the tested organisms (*E.coli*, *S.Typhimurium*, *Enterobacter cloacae* or *Serratia marcescens* ) separately without addition of OA and served as control group, while the four other groups were inoculated with OAs and test organism as follow;

Group 2: one of the test organism + AA 5000 ppm

Group 3: one of the test organism + LA 10000 ppm

Group 4: one of the test organism + LA 5000 ppm + CA 5000 ppm

Group 5: one of the test organism + MA 10000 ppm + CA 5000 ppm

After mixing; all groups were left at room temperature until complete coagulation, whey was drained using sterile gauze, curd was then placed under light pressure and kept overnight at 20 ° C for complete whey drainage. The resultant cheeses were stored in stomacher bags at 10° C for 9 days for bacterial enumeration

**Evaluation of the OAs' inhibitory effect against tested EB in fresh Tallaga cheese**

The antibacterial effect of OAs in cheese matrix was evaluated by enumerating the inoculated bacterial strains using selective media and counting TVC on TSA (BD) media on

day 0 (after complete whey drainage), 3, 6 and 9. Ten grams of each cheese sample were diluted in a stomacher bag with 90 ml of sterile saline solution and homogenized for 30 s at 230 rpm, after that they were subjected to further serial dilution with sterile saline solution to achieve the desired concentrations, from which 100 µl were plated onto duplicate plates of chromocult coliform agar "CA" (Merck) for *E.coli*, DHL (EIKEN) for S.*Typhimurium*, and Violet Red Bile Glucose Agar "VRBG" (Oxoid) media for both *Enterobacter cloacae* and *Serratia marcescens*. Plates were incubated at 37° C for 24 h and bacterial colonies were counted, while for counting TVC; TSA agar plates inoculated with diluted samples of all groups were incubated at 30° C for 48 h.

**Organoleptic properties**

The effect of adding OAs to Tallaga cheese on its sensory characteristics was evaluated by at least 10 panelists at three points during the storage period (day 0, 5 and 9). Five test samples (cheese without OAs, cheese with 5000 ppm of AA, cheese with 10000 ppm of LA, cheese with 5000 ppm LA +5000 ppm CA and cheese with 10000 ppm MA+ 5000 ppm CA) were evaluated for their Appearance (body &texture), smell, taste and overall acceptance on a 5-point scale system as follow: 1 = Bad, 2 = Somewhat bad, 3 = Normal, 4 = Somewhat good and 5 = good. Each parameter's value was shown as mean value of all determinations.

**Physiochemical properties**

pH

pH of both control and test groups was measured at day 0 using digital pH meter

Water activity "$a_w$"

A negative control cheese (cheese without tested bacteria or OA) was manufactured and stored at 10° C for 9 days, from which $a_w$ was measured at day 0, 3, 6 and 9 using AQUALAB 4TE water activity meter ( METER Group, Inc. USA).

**Statistical analysis**

All experiments were carried out in triplicates (n=3), and the average of results was calculated and shown.

## Results and Discussion

### Inhibitory effect of OAs against EB in fresh Tallaga cheese

In this section the antimicrobial effect of four OAs against four different types of EB including hygienic indicator "*E.coli*", pathogenic "*S. Typhimurium*", opportunistic "*Enterobacter cloacae*" and causative cheese defect "*Serratia marcescens*" was evaluated in laboratory manufactured Egyptian Tallaga cheese. Four times MIC values of AA, LA (according to the lowest MIC value against examined strains in vitro) and combination of LA+CA and MA+CA (according to their MIC in a synergistic action based on FIC calculation) were inoculated into cheese due to the known contradictory effect of cheese proteins and fat content as intrinsic factors which require a higher concentration of antibacterial agent than that used in laboratory culture media (Moro et al., 2013). AA and LA were found to be the most effective with the lowest MIC values of 1250 and 2500 ppm respectively against EB strains in the culture media, and two combinations of LA 1250+ CA 1250 and MA 2500+CA 1250 ppm have shown a good synergistic effect based on their FIC values, for that reasons they were selected for the study in cheese matrix.

The growth of *E.coli* in control sample of cheese has increased through the storage period reaching its maximum count at day 6 (8.2 log CFU/g) and then slightly decreased to 7.8 log CFU/g at the end of storage period (9 days) on selective agar media (fig. 22), while applying OAs singly and in combinations showed a strong inhibitory effect against *E.coli* from day 0. For all treatment conditions "AA 5000ppm (0.5%), LA 10000ppm (1%), LA 5000ppm+CA 5000ppm (0.5%+0.5%), MA 10000ppm+CA 5000 ppm (1%+ 0.5%)"; the bacterial count was under the detection limit (3 log CFU/g) where the strongest bactericidal effect was shown in case of combining 10000ppm of MA with 5000 ppm of CA.

In case of *S. Typhimurium*, the bacterial count increased over 6 days from 6.5 log CFU/g at day 0 to 7.1 log CFU/g at day 6, then slightly decreased again at day 9 to 6.2 log CFU/g in control sample (Fig. 22), while *S. Typhimurium* growth was suppressed when 0.5% of AA was

used by 2.3 log CFU/g at day 0 compared to control sample, and complete inhibition was achieved by day 3 in case of AA and from day 0 in case of other treatments (LA 1%, LA 0.5% +CA 0.5% MA 1%+CA 0.5%).

For *Enterobacter cloacae*, in case of control cheese, the bacterial count slightly increased to 7.3 log CFU/g at day 3 and then decreased to 6.4 log CFU/g at day 6 and then reached 6.8 log CFU/g at the end of storage period (Fig. 22). When the three treatments of AA 0.5 %, LA 1% and MA 1%+CA 0.5% were applied the bacteria could not be detected from day 0 along the storage period, However *Enterobacter cloacae* showed some resistance to a combination of LA 0.5% +CA 0.5% where the bacterial growth was suppressed by more than 2 log CFU/g at day 0 and 3, then started to decrease under the detection limit from day 6 until the end of the storage period.

As for *Serratia marcescens*, the bacterial count increased in control group reaching 8.7 log CFU/g by day 9 (Fig. 22), when cheese was treated with different OAs, the bacteria could not be detected from day 0 until the end of storage period, where LA 1% and MA 1%+CA 0.5% showed complete bactericidal action against *Serratia marcescens*.

Growth of mesophilic bacteria for all tested groups was assessed by enumeration on TSA (BD) media, results showed that TVC was below the detection limit for all kinds of treatments over the study period in case of *E.coli*, *S.Typhimurium* and *Serratia marcescens* where the TVC count of control groups increased to 8.4, 7.7 and 8.9 log CFU/g respectively. In contrast, TVC of the cheese inoculated with *Enterobacter cloacae* was not completely inhibited when LA 0.5% +CA 0.5% were applied until day 6, but the total volatile count was dramatically decreased than the count of control cheese by 4.1, 3.8 and 4.5 log CFU/g at day 0, 3 and 6 respectively, then was under the detection limit at day 9, for the same bacteria when LA 1% was added, the TVC count was under the detection limit until day 6 and then detected at day 9 with at a low concentration of 3 log CFU/g, other OAs (AA 0.5% and MA 1%+ CA 0.5%) caused the total volatile bacteria to be completely suppressed over the storage period.

Attempts to inhibit the undesirable growth of several bacterial species in cheese including Egyptian cheese are numerous, owing to their ability as a rich-protein food, pH range and water activity to support the growth of pathogens. Tallaga cheese is one of the most popular

soft cheeses in Egypt that gains a high consumer demand and acceptability, furthermore this cheese could be used as a new functional dairy product that supports human health (El-Kholy et al., 2016), unfortunately, in the first part of this study it was demonstrated that the microbial load of Egyptian cheeses was considered high in terms of TVC, EB and *E.coli*, in particular, Tallaga cheese harbored a considerable count of EB including *E.coil* (Table 1), therefore, improving safety of Tallaga cheese was taken into account in the last part of the study by inhibiting the growth of different EB strains, for this purpose, OAs as safe biopreservatives have been employed to achieve the desired microbial reduction.

Enterobacteriacae is an important bacterial family with a significant importance to food industry specially milk and its products (Mladenović et al., 2021), their inevitable entrance to food chain is regarded to their ubiquitous distribution in the nature as a large group of different bacterial genera, this family includes at least 48 genera, 219 species and 41 sub-species with different degrees of harmness when considering human health (Baylis et al., 2011), in this study four strains were chosen to cover different aspects including pathogenic, opportunistic, spoilage and hygienic impact of members of EB group.

*E. coli* species includes different strains, although some of them are harmless, they gain a particular interest in food industry as a hygienic indicator for fecal contamination that should not be found in cheese when basic GMP is followed (Metz et al., 2020; Baylis et al., 2011). Salmonellosis foodborne illness is one of the serious diseases that affects human health worldwide. In 2020, outbreaks caused by Salmonella in Europe has been primarily linked to consumption of cheese contaminated with the bacteria (Napoleoni et al., 2021), furthermore, a frequently reported prevalence of *Salmonella* spp. in Egyptian dairy market (El-Baz, 2017; Ibrahim et al., 2015) is still representing a major challenge. Recently cheese was reported to be the main source of different Salmonella serovars including *S. Typhimurium* with multidrug resistance capability in a study of the dairy products in Egypt (Elafify et al., 2022).

*Entrobacter* spp. and *Serratia marcescens* are among genera of EB family that have been isolated from traditional cheese (Mladenović et al., 2018) and their ability to form biofilm on the milk pipes stainless steel surface of dairy plant was indicated (Cherif-Antar et al., 2016). *E. cloacae* is an emerging pathogen that caused neonatal sepsis with a considerable mortality

rate within the first 72 hours after birth as the second common etiological agents over 20 years in south Korea (Song et al., 2021), powdered infant formula (PIF) is a sensitive dairy product that is suggested to be a source of foodborne infection by *E. cloacae* (Estuningsih et al., 2006). *S. marcescens* is also considered as another emerging cause of infection in hospitalized persons and infants, besides being an opportunistic pathogen, its presence in dairy products (Amorim et al., 2018) and other food (Mohanram et al., 2020) can cause spoilage as well as lowering the product's quality owing to its ability to form a red pigmentation on food surface.

Although Enterobacteriacae as a gram-negative bacteria are generally more resistant to the action of antimicrobial compounds than gram- positive bacteria due to the complex structure of its outer cell membrane (Nazzaro et al., 2013; Raftari et al., 2009) in addition to the water acivity of Tallaga cheese (0.97) shown in Table 12. which is supporting the growth of EB that usually grow at the best above $a_w$ 0.95 in , Our results showed that OAs are strong antibacterial agents which completely controlled the growth of selected EB strains in Tallaga cheese over 9 days of incubation at 10° C, a decrease in pH that is shown in Table 11. is a main factor that contributed to OAs antimicrobial effect specially when the pH is very close to its acid constant (Stratford and Eklund, 2003) where the equilibrium between the undissociated and dissociated form of the acid is achieved leading to penetration of bacterial cell membrane by the undissocaited OA and subsequent cell death (Yasothai and Giriprasad, 2015). In accordance to our results a previous report stated that organic acids like malic, formic, propionic, citric and acetic acids were an effective antimicrobials to inhibit the growth of *E. sakazakii* where propionic and acetic acids had the highest effect in liquid foods (Back et al., 2009). In line with that, Zhu et al., (2013) also provided the idea of using acidified milk formula to control the colonization and translocation of E. sakazakii. In cheese, ascorbic acid in a concentration reached 3% was needed to reduce the bacterial count of *S. Enteritidis*. below 3 log CFU/g in soft Egyptian cheese with 3% of salt following 3 weeks of incubation at 4° C (Elafify et al., 2022), while our results indicated that other OAs like AA, LA, MA and CA might be more effective in controlling EB in cheese since a lower concentration at 0.5 up to 1.5 % had a strong bactericidal effect for cheese incubated at 10° C. Furthermore the use of OAs like acetic, lactic and citric in meat preservation has been extensively employed to control undesirable bacteria including EB (Ben Braïek and Smaoui, 2021).

Of all OAs evaluated in literature, acetic and lactic acid are found to be the most acceptable (Yasothai and Giriprasad, 2015a)

OAs in this study showed a bactericidal effect for examined EB from day 0 except for AA in *S. Typhimurium* and LA+CA in *E. cloacae* that took 3 and 9 days respectively to reach the complete bactericidal effect, the EB has the ability to adapt and grow at a pH concentration between 3.8 and 9, thus the possibility of EB to develop an acid-tolerant mechanism in acidic food should be controlled (Alvarez-Ordóñez et al., 2015), furthermore, the nature of acid and the type of target organism are among factors that many cause a variation in the antimicrobial efficacy by an acid (Rajkovic et al., 2010).

**Organoleptic properties of cheese made with OAs**

In the current work, panelists have evaluated the fresh cheese based on four sensory attributes (body &texture, smell, taste and overall acceptance) over the storage period. Results are shown in Fig. 24. At day 0 the control sample recorded the highest score within all examined cheeses for all sensory parameters, however the cheese texture was the most non affected parameter in all treatments compared to control cheese, and other parameters were still acceptable except for the taste and overall acceptance of cheese treated with malic and citric acids in combinations that recorded 2.45 and 2.8 respectively due to the feeling of strong sour taste resulted from the high acid concentration (1.5 % in total). In contrast, the cheese body and texture was improved in almost all the treated samples over the storage period at day 5 and 9 compared to the control sample which showed a decrease in all parameters at day 5 and 9 comparing to day 0 except for the smell where the control cheese recorded the best score (3.9) at the end of storage period together with cheese treated with lactic acid 1%. At day 5 the cheese taste score was slightly higher (3.7) for the control sample than samples treated with LA and LA+CA (3.5 each), however the overall acceptability was almost same for three of them scoring 3.5, 3.5 and 3.4 respectively, the latter two parameters were slightly not acceptable for treatments with AA and MA+CA which recorded score lower than 3. Interestingly, as the sensory parameters of the control samples were generally decreased at the end of storage, at day 9, treatments with AA and LA showed the highest scores specially for the taste (3.8and 4.2 respectively) and overall acceptability (3.8and 4.1 respectively) than the control sample that

recorded 3.4 and 3.5 for the two parameters respectively, while treatment with LA+CA was generally accepted with scores between 3.2 to 3.4 for all four parameters, however cheese containing MA+CA remained with the lowest acceptability for taste (2) and overall acceptance (2.5).

In general; the cheese treated with OAs were well accepted by the panellists, especially for the cheese fortified with lactic acid that offered the most pleasant taste and good smell, particularly at the end of storage time when its acceptability exceeded that of the control sample, on the contrary, cheese treated with a combination of malic and citric acids tasted somewhat bad due to its distinct sour taste until the end of storage period.

Among factors that limit the application of OAs into food material, their possible negative impact on sensory attributes poses some challenge specially when high concentration of acid is needed to achieve the low pH necessary for the antibacterial activity (Mani-Lopez et al. 2012), that negative impact was just noticed in case of combining malic and citric acid with a final concentration of 1.5% in our study.

Although cheese sensory properties may be affected by incorporating antibacterial agents, some studies suggested that regardless of cheese appearance and texture that were improved by adding antimicrobials during storage period, cheese flavor score was decreased but was higher than control sample at the last half of the storage period due to deterioration of the later one (Ahmed et al., 2021). Accordingly, our study also suggested that some treatments as in case of Lactic acid has improved the cheese sensory properties, some panelists have pointed out the distinct pleasant taste of this cheese particularly, this preference of cheese treated with LA may be attributed to the similarity to common flavor of cheese developed naturally by LAB in fermented cheese. Furthermore, panelists commented that the cheese treated with OAs had a more salty taste than control sample, this finding might be useful for making a healthier cheese in the future by decreasing the percentage of salt and adding OAs to achieve the desired taste.

**Physiochemical properties of Tallaga cheese**

pH and water activity

Results of three replicates measurement of pH value of control and test cheeses is shown in Table 11. Water activity of Tallaga cheese at day 0 measured 0.973, this value remained constant (0.97) along the other 3 measurements at day 3, 6 and 9 (Table 12).

In chapter 3, the antimicrobial activity of different OAs against four EB strains was investigated in Tallaga cheese incubated at 10° C for 9 days following a determination of their MIC and FIC values in section 1, the lowest MIC values were belonging to acetic and lactic acids respectively, while citric and malic acid had a lower MIC values that more than 10000 of ppm those two acids were not able to inhibit some EB in vitro, however a strong synergistic effect of MA+CA confirmed with FIC value of 0.5 and 0.375 against *E. cloacae* and *S. marcescens* respectively has been shown in vitro, another synergism of combining LA and CA against *S. Typhimuruim* was also noticed (FIC = 0.5), the latter two species showed a strong resistance to OAs comparing to other examined strains in vitro, thus they were chosen with *E.coli* and *S Typhimurium* for the study in cheese . In cheese matrix the antimicrobial effect of tested OAs was confirmed for all EB examined, however *S. Typhimurium* and *E. cloacae* showed a slight resistance to AA and LA+CA respectively which declined after 3 and 9 days respectively reaching a complete bactericidal effect. The pH range and $a_w$ measurements were supportive to the study, pH of OAs were close to their pka and the$a_w$ enabled the growth of EB in cheese. The sensory test elaborated that OAs can be used as an effective intervention to control different strains of EB including pathogenic and harmless bacteria in soft cheese with a good acceptability with exception of MA+CA that gained the least sensory acceptability for the panelists. Although OAs are usually formed naturally during the fermentation of food products including cheese, the direct or indirect addition of the chemically synthesized OAs to food are also applicable (Stratford and Eklund, 2003).

**Table 11: pH values of cheese treated with different OAs**

| EB strain | pH value | | | | |
|---|---|---|---|---|---|
| | control | AA | LA | LA+CA | MA+CA |
| *E. coli* | 6.2 | 4.9 | 3.9 | 3.5 | 3.0 |
| *S. Typhimurium* | 6.4 | 4.2 | 3.5 | 3.8 | 3.4 |
| *E. cloacae* | 6.1 | 4.2 | 3.6 | 3.5 | 3.2 |
| *S. marcescens* | 6.1 | 4.3 | 4.0 | 3.7 | 2.5 |

Values are mean of three replicates SE ± (n=3)

**Table 12: water activity of cheese during the storage period.**

| Time (day) | water activity |
|---|---|
| 0 | 0.973 |
| 3 | 0.97 |
| 6 | 0.97 |
| 9 | 0.97 |

Tallaga cheese preparation

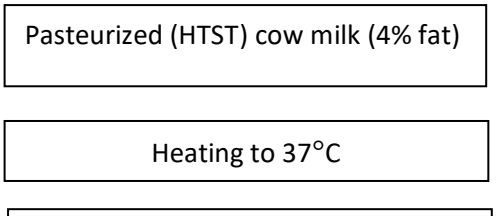


**Figure 23** Illustration of Tallaga cheese making for examining the effect of OAs against EB.

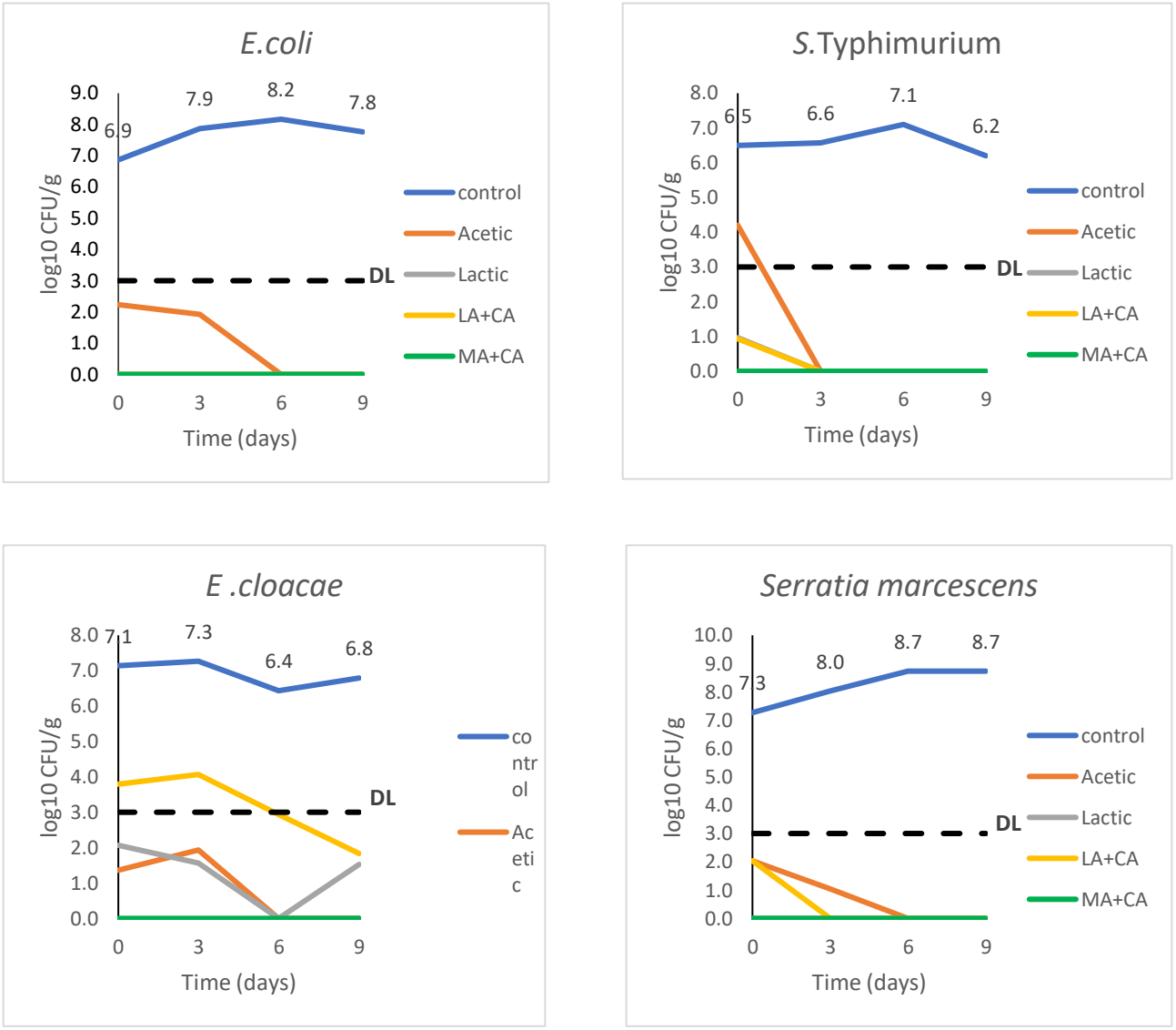



**Figure 24** Antibacterial effect of organic acids against tested EB strains: blue (control), orange (acetic acid 0.5%), gray (lactic acid 1%), yellow (lactic acid 0.5% + citric acid 0.5%), and green (malic acid 1% + citric acid 0.5%) on selective media. Dotted line shows the detection limit (DL). Values are mean (n=3).

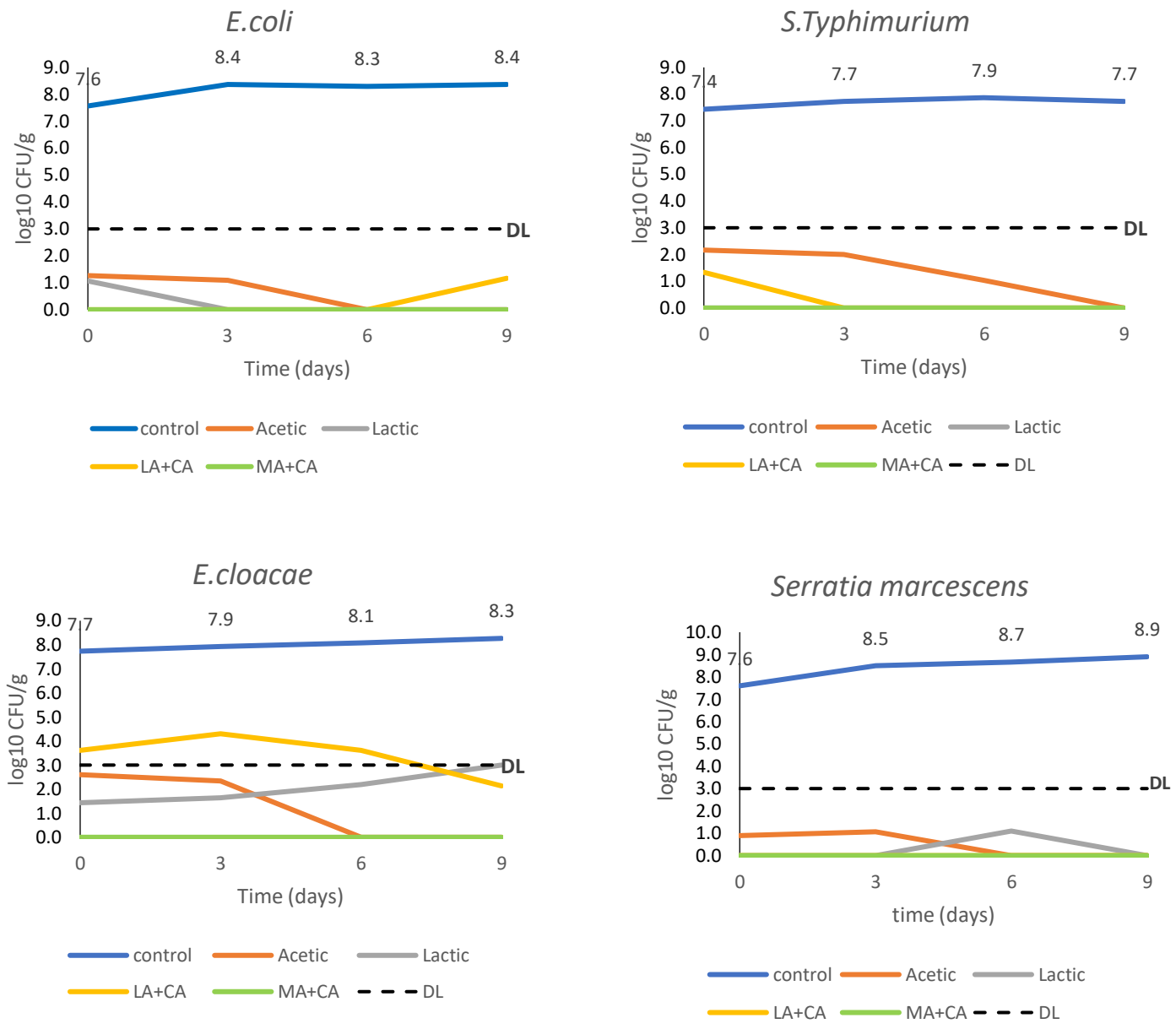



**Figure 25** TVC of cheese inoculated with EB strains without OAs /control (blue) or with AA acetic acid 0.5% (orange), lactic acid 1% (gray), lactic acid 0.5% + citric acid 0.5% (yellow), and malic acid 1% + citric acid 0.5% (green). . Dotted line shows the detection limit (DL). Values are mean (n=3).

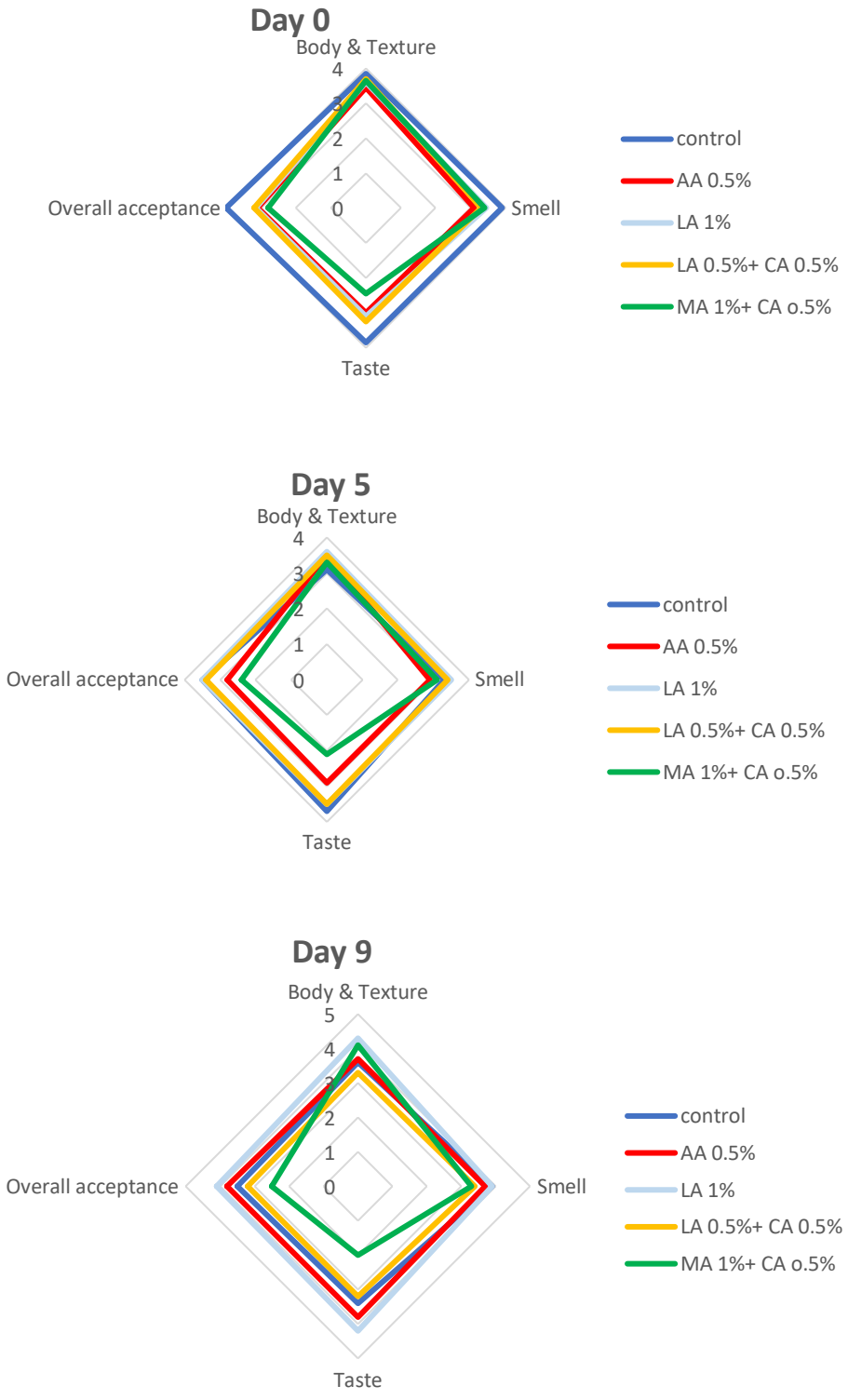



**Figure 26** Impact of adding OAs to cheese on its sensory properties including body & texture, smell, taste and overall acceptability over the storage time.

# Chapter 4: General Conclusion

The fact that Egypt provides the first archeological evidence of cheese production since more than 3200 B.C. has attracted many Egyptian researchers to understand the production and safety of this variety of cheese, however due to shortage resources and technological facilities, most studies have focused on using classical methods for those purposes. Moreover, studies concerning the deeper understanding of other bacterial communities in cheese that contributes to functional and nutritional properties of cheese and confer various health benefits through probiotic application rather than the pathogenic bacteria have been greatly revolutionized since the release of NGS in 2005.

Since cheese is as PHF that have been implicated in many cases of food born outbreaks, the first objective of this study was to evaluate the level of contamination of cheese. In chapter 2/ section 1, 19 different samples of 6 cheese types were collected and analyzed by culture-dependent and culture-independent methods, the results highlighted the need to implement a strict hygienic measures and effective GMP during all stages of cheese production, storage and handling, since detectable viable bacterial counts of different species usually originated from processing environment including working staff, on the other hand the prevalence of significant load of undesirable organisms responsible for cheese spoilage and various defects reflects an inferior quality of the cheese and possible product loss.

In chapter 2/ section 2, the biodiversity of the bacterial microbiota of Egyptian cheeses was deeply understood through 16S rRNA Amplicon sequencing. Generally; Egyptian cheese appeared rich and diverse in bacterial communities from phylum to species level, while diversity index had a moderate value comparing to other cheese types. The ten major genera that were present in more than 74% of cheese samples made up the core microbiota of the cheese (mainly LAB) indicating that they are responsible for developing the distinctive taste of Egyptian cheese despite their relative abundance that varied between cheese types as shown in Fig. 10. Unfortunately, part of them arec considered problematic in cheese environment, e.g. Enterobacteriaceae and pseudomonas, the former indicates bad hygiene in consistence with

finding in chapter 1, the later is responsible for various kinds of cheese defects, spoilage and biofilm formation.

Results of this study has confirmed the previous reported fact that NGS analysis provides deeper and complete understanding of all bacterial genera including minor and subdominant species (Alegria et al., 2012; Dalmasso et al., 2016) that was also clear when comparing the result of NGS method in Domiati cheese with the previously reported results by El-Baradei et al. (2007) using other culture-independent methods.

Since this is the first time to analyze Egyptian cheese by this robust technique, several genera that have been detected for the first time in Egyptian cheese such as *Lysobacter, pseudomonas, Chryseobacterium, marinilactibacillus, Weisella, Enhydrobacter, Planococcaceae Tetragenococcus, Corynebacterium, halanaerobium Kocuria* and others. Interestingly; 4 genera not previously associated with cheese have been observed in the present study including *Lysobacter* that was a part of core microbial composition existed in all 19 examined samples, *Salinivibrio*, *Alteromondales* and *Candidatus* Portiera, the latter is associated with pest infestation.

While NGS could assign 101 sequence on the species level, the existence of some undesirable species such as *S. epidermidis, S. aureus, B. cereus* and *Propionibacterium acne*s suggests several risks on manufacturing environment such as biofilm formation, and on consumption of cheese even those made from pasteurized milk due to the possibility of existence of heat stable toxins, spores or transfer of antibiotic resistance genes.

PCA analysis suggested that three patterns may be responsible for the composition of bacterial microflora in Egyptian cheese; Cheese type, salinity and manufacturing technique, the majority of the chees types could group based on their type, except Domiati cheese that highlight the importance of standardization of processing techniques between different dairy producers to obtain a harmonized product with specific characteristics, in general we can conclude that no particular element could be decisive for the composition of the microflora in this complex environment, a set of factors from farm to fork with relevance to industry were previously discussed in that regard (Montel et al., 2014; Marino et al., 2017; Jonnala et al., 2018).

The argument regarding traditional cheese between being recognized as a treasure for their richness and diversity of microbiota responsible for its distinctive sensory properties and between the possible contribution to food poisoning with increasing the risk associated with pathogenic bacteria that may present in raw milk used for traditional cheese manufacturing is still not solved, the composition of raw milk may exhibit a potential benefits against some pathogenic bacteria including *E.coli*, *Staphylococcus aureus*, and *Listeria monocytogenes* , while on the other hand traditional practices and using raw milk for cheese production without strict managerial programs to guarantee the safety of unpasteurized cheeses can be implicated in several food-borne diseases (Montel et al., 2014).

To understand the possible factors that contribute to the Egyptian cheese's microbial composition further studies is recommended to include different parameters such as production line, milk treatment, use of starters, PH, production date and season , since Identification of dominant bacteria responsible for organoleptic characterization of cheese by HTS can help producers to choose the production method to obtain specific and standardized characterization, minimize variation within same kind of cheeses and enhance the cheese quality as well as safety (Guidone et al., 2016).

In order to provide a cheese with high safety parameters and suitable for human consumption, minimizing the risk of food borne diseases associated with eating cheese was taken into consideration. The high incidence of EB in Egyptian cheese was evidenced by the viable cell count and further confirmed by culture independent method in chapter 2, the EB family includes a wide range of bacteria with a particular potential impact on cheese safety and quality and the control of EB in cheese is a persistent challenge in developing countries. Different bacteria belonging to EB group were studied in chapter 3 for their susceptibility to the action of different OAs. All tested bacterial species were inhibited by at least one OA in vitro, the MIC of each OA varied according to the tested microorganism, furthermore; OAs have proved no inhibitory effect for LAB in vitro that are essential to cheese as a fermented product in addition to exhibiting synergistic effects of different combinations against some of the tested EB group but none of LAB.

Incorporation of OAs to cheese matrix for the aim to assess their antibacterial effect against EB inside the cheese matrix was carried out in the last step, four different EB strains were chosen to cover different aspects related to the importance of EB group being including pathogenic, opportunistic and harmless bacteria. OAs and their various combinations seemed to be safe and effective antibacterial agents that can be used as a part of hurdle technology to ensure the overall safety of cheese products. Furthermore; their minimal effect on sensory characteristics of cheese nominate them as a good alternative for unwanted practices in cheese manufacturing.

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