AMERICAN UNIVERSITY OF BEIRUT

ASSESSMENT OF THE MICROBIOLOGICAL ACCEPTIBILITY OF AKKAWI CHEESE IN BEIRUT AND THE ANTIMICROBIAL RESISTANCE PROFILES OF ASSOCIATED *ESCHERICHIA COLI*

by
NASRI HASSAN DAHER HUSSEIN

A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
to the Department of Nutrition and Food Sciences
of the Faculty of Agricultural and Food Sciences
at the American University of Beirut

Beirut, Lebanon
April 2022

AMERICAN UNIVERSITY OF BEIRUT

ASSESSMENT OF THE MICROBIOLOGICAL ACCEPTIBILITY OF AKKAWI CHEESE IN BEIRUT AND THE ANTIMICROBIAL RESISTANCE PROFILES OF ASSOCIATED *ESCHERICHIA COLI*

by

NASRI HASSAN DAHER HUSSEIN

Approved by:

Signature

Dr. Samer Kharroubi, Associate Professor — Advisor
Department of Nutrition and Food Sciences

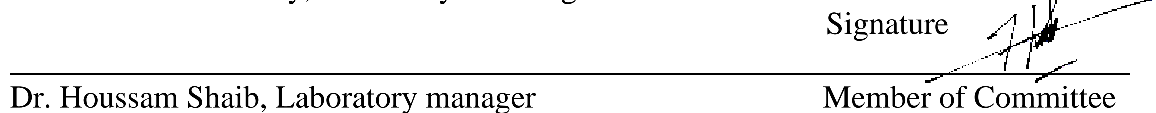
Signature

Dr. Imad Toufeili, Professor — Member of Committee
Department of Nutrition and Food Sciences

Signature

Dr. Issmat I. Kassem, Assistant Professor — Member of Committee
Center for Food Safety, University of Georgia

Signature



Dr. Houssam Shaib, Laboratory manager — Member of Committee

Date of thesis defense: April 29, 2022

AMERICAN UNIVERSITY OF BEIRUT

THESIS RELEASE FORM

Student Name: __Daher Hussein ____ Nasri ____________ Hassan_
Last First Middle

I authorize the American University of Beirut, to: (a) reproduce hard or electronic copies of my thesis; (b) include such copies in the archives and digital repositories of the University; and (c) make freely available such copies to third parties for research or educational purposes:

☐ As of the date of submission

☐ One year from the date of submission of my thesis.

☒ Two years from the date of submission of my thesis.

☐ Three years from the date of submission of my thesis.

____________________________________May 11, 2022_____________

Signature Date

ACKNOWLEDGMENTS

I would first like to express my sincere appreciation and gratitude to my thesis advisor Dr. Issmat Kassem who was there for me and guided me every step of the way, even when abroad. Dr. Kassem has been my professor since I was an undergraduate student and I owe my introduction to the word of microbiology to him. I would also want to express my sincere appreciation to my thesis committee members Dr. Samer Kharroubi, Dr. Imad Toufeili, and Dr. Houssam Shaib for their continuous support. Dr. Kharroubi and Dr. Toufeili were also my professors as an undergraduate student. Dr. Kharroubi introduced me to different concepts in the rich field of biostatistics and assisted me whenever I had inquiries related to this field. Dr. Toufeili taught me valuable knowledge related to the chemical and biochemical composition of different types of food. As for Dr. Shaib, I made his acquaintance when I started my thesis lab work and I want to thank him for sharing with me his valuable expertise in lab techniques and in the field of immunology. It was both an honor and a pleasure having you as members of my thesis committee.

I also, want to take the time to express my sincere appreciation to the previous lab supervisor Ms. Tsolaire Sourenian who shared her expertise with me and guided me in my lab work when I first entered the microbiology laboratory. I would also, like to thank my colleagues and lab mates Sarah El Khechen, Sarra Grazza, Abdallah Alhaj Sulaiman, and Ali Assi for their aid and support during my time in the lab. I am grateful for the memories I share with them in the lab. I am also grateful for the acquaintance of lab mates and colleagues Mona Zeidan and Léa Dagher. In addition, I am grateful for the acquaintance of colleague Ms. Carla Mallah.

I would also, want to thank Dr. Issam Bashour for granting me the permission to test the electric conductivity of my brine samples in one of the labs of the department of agriculture. I would also want to thank the departmental research assistant in the department of agriculture Ms. Razan Dbaibo and graduate research assistant Mr. Imad Keniar for assisting me in testing the brine samples.

I would also, like to thank the departmental research assistant in the department of nutrition and food sciences Ms. Hala Fallah for keeping up with us and taking the charge of ordering new lab materials whenever resources became scarce.

I would also, want to thank the new lab supervisor Ms. Razan Zeineddine for her support when it came to different inquiries related to the lab.

Lastly, I would like to thank my parents and my brother Fares for their everlasting love, care, and support that keep me going. I would also like to thank my friends for their constant support.

Finally, I would like to dedicate my work to every individual seeking science to learn, grow, and help the world become a better place.

Thank you.

ABSTRACT
OF THE THESIS OF

Nasri Hassan Daher Hussein for Master of Science
Major: Food Safety

Title: Assessment of The Microbiological Acceptability of Akkawi Cheese in Beirut and the Antimicrobial Resistance Profiles of Associated *Escherichia Coli*

Lebanon, like many other developing countries, suffers from the toll of emerging foodborne pathogens and antibiotic resistance within its food systems. Very little data is collected on the presence of foodborne pathogens in different types of food. Furthermore, research on the emergence and dissemination of antibiotic resistance within different food systems remain limited. For that purpose, this study focused on Akkawi cheese a popular local food item. The objective of the study was to evaluate the microbiological acceptability of Akkawi cheese from different retail stores in Beirut. This was done by assessing the densities of *Escherichia coli* and fecal coliforms, which are indicators of fecal pollution, as well as *Staphylococcus aureus*, which is an indicator of hygiene. The objective of the study was also to assess the antibiotic resistance phenotypes of the isolated *E. coli.* 50 Akkawi cheese samples and 13 brine samples were collected from different retail stores from 9 different districts of Beirut. A total of 135 *E. coli* strains were isolated: 118 (87.4%) were isolated from the cheese samples and 17 (12.6%) were isolated from the brine samples. Antimicrobial susceptibility testing, using the disk diffusion assay, revealed overall resistance of *E. coli* to penicillin (100%), ampicillin (68.1%), amoxicillin-clavulanate (88.1%), cefepime (21.5%), cefotaxime (43.0%), cephalexin (63.0%), cefixime (18.5%), doripenem (34.8%), meropenem (34.8%), imipenem (17.0%), gentamicin (40.0%), kanamycin (35.6%), streptomycin (57.8%), tetracycline (40.7%), ciprofloxacin (4.4%), norfloxacin (4.4%), trimethoprim-sulfamethoxazole (46.7%), and chloramphenicol (28.2%). But also, of the 135 *E. coli* isolates, 101 isolates (74.8%) were multidrug resistant (MDR). Furthermore, 2 *mecA* methicillin-resistant *Staphylococcus aureus* (MRSA) strains were detected in one of the cheese samples. These findings revealed critically high levels of multidrug resistance in Lebanese food systems. Multidrug resistant infections are on the rise, and they are difficult to treat using conventional antibiotics. And the fact that high levels of multidrug resistant *E. coli* were detected, means that other multidrug pathogens are likely to be present in dairy food systems as well as other food systems. The presence of high levels of multidrug resistance within Lebanese food systems contributes to an effective dissemination and circulation of antibiotic resistance within the community. Failure to act fast will result in a major public health crisis.

TABLE OF CONTENTS

ACKNOWLEDGMENTS 1

ABSTRACT 3

ILLUSTRATIONS 6

TABLES 7

INTRODUCTION 8

MATERIALS AND METHODS 22

A. Sample Collection 22

B. Culture and Isolation of Escherichia coli and Staphylococcus aureus 22

C. Antimicrobial Resistance (AMR) 23

D. Measurement of pH and Salinity of Brine Samples 24

E. Polymerase Chain Reaction (PCR) 25

1. For *Escherichia coli* 25

2. For *Staphylococcus aureus* 26

F. Statistical Analysis 27

RESULTS 28

A. Bacterial Densities 28

1. Bacterial Densities in Cheese Samples 28

2. Bacterial Densities in Brine Samples 30

C. *Staphylococcus aureus* PCR Confirmation and Methicillin-resistant Strains Identification 45

DISCUSSION.. 47

CONCLUSION 52

REFERENCES 54

ILLUSTRATIONS

Figure

1. *Escherichia coli* and fecal coliform densities (log_{10}CFU/g) in Akkawi cheese samples 29

2. *Staphylococcus aureus* and other staphylococci densities (log_{10}CFU/g) in Akkawi cheese samples 30

3. *Escherichia coli* and fecal coliform densities (log_{10}CFU/ml) in Akkawi brine samples 31

4. *Staphylococcus aureus* and other staphylococci densities (log_{10}CFU/ml) in Akkawi brine samples 31

5. Percentage of antimicrobial resistance to different antibiotics amongst *Escherichia coli* isolates from Akkawi cheese and the collected brine 34

6. PCR signals for the *femB* gene 45

7. PCR signals for the *mecA* gene 45

TABLES

Table

1. Correlation coefficients (R) from Pearson correlation analysis between the brine *Escherichia coli* and fecal coliform densities and the cheese *Escherichia coli* and fecal coliforms densities, as well as the pH, and salinity (g/L) of the brine and from Pearson correlation analysis between the cheese *Escherichia coli* and fecal coliform densities and the pH and salinity (g/L) of the brine 32
.... **Error! Bookmark not defined.**

2. Correlation coefficients (R) from Pearson correlation analysis between the brine *Staphylococcus aureus* and other staphylococci and the cheese *Staphylococcus aureus* and other staphylococci, as well as the pH, and salinity (g/L) of the brine and from Pearson correlation analysis between the cheese *Staphylococcus aureus* and other staphylococci and the pH and salinity (g/L) of the brine 32

3. Prevalence of the phenotypical antimicrobial resistance of *Escherichia coli* isolated from Akkawi cheese and the collected brine 35

4. Prevalence of phenotypic resistance of *Escherichia coli strains* from the Akkawi cheese samples and from the brine samples 37

CHAPTER I

INTRODUCTION

Lebanon is considered a developing country and is located in the Eastern Mediterranean region, that is ranked third amongst regions with the highest estimated burden of foodborne diseases (Kharroubi et al., 2020). And thus, food safety is amongst the various significant challenges Lebanon faces. And like many other developing countries, Lebanon suffers from the toll of foodborne illnesses on its public health and economy and lacks a sustainable food safety system that allows the control of food contamination and the mitigation of foodborne illnesses (Kharroubi et al., 2020). In November 2014, however, food safety took a right turn. In fact, surprise inspections of restaurants, slaughterhouses, supermarkets, and farms were conducted at the behest of the Lebanese Ministry of Public Health (World Health Organization [WHO]). More than 1,000 establishments were named by the Minister of Public Health at the time, Wael Abou Faour, for unsatisfactory food sampling or inspection results (World Health Organization [WHO]). This even resulted in some of these establishment to be shut down (World Health Organization [WHO]). This created an increased sense of awareness amongst actors in the food chain and consumers. Consumers became more alert to the food products they consider purchasing (World Health Organization [WHO]). As for establishments, they started working on reinforcing their internal food safety policies and started seeking international food safety certification from the International Organization for Standardization (World Health Organization [WHO]). Furthermore, this initiative encouraged a closer collaboration between the Ministry of Public Health and other ministries including the Ministry of Economy and Trade (World Health

Organization [WHO]). This initiative also allowed the reactivation of the food safety that has already been drafted and presented it to the Lebanese parliament for approval (World Health Organization [WHO]). Despite this progress, food safety in Lebanon remains basic and flawed. For instance, the food safety page on the Ministry of Public Health's official website presents very limited insight on important aspects of food safety. In fact, the four main headlines of the page consist of the "Food Inspection Checklist", the "Food Sampling Form", a section entitled "Food Inspection" which only present bullet points of the requirements for food safety in food institution (Ministry of Public Health [MoPH]). Furthermore, one the headlines was "WHO: The Five Keys to Safer Food Programme", which provides very basic food safety guidelines of which some are related to cooking and storing food at adequate temperatures, without at least providing the adequate cooking and food storage temperatures (Ministry of Public Health [MoPH]). But also, the latter provided general information about foodborne illnesses without even specifying the responsible agents. This reveals very limited knowledge related to food safety and causative agents from the part of the Lebanese government. This can be reflected in the study by Kharroubi et al., which compiled and analyzed the microbiological data that were based on the food safety campaign that was launched in 2015 by the Ministry of Public Health. Out of the 11,625 tested food samples a total of 3,334 food samples were found to be microbiologically unacceptable based on the LIBNOR standards, which is a relatively high percentage of 28.7% of the entirety of the samples (Kharroubi et al., 2020). Amongst the tested food categories, dairy had a notably high number of microbiologically unacceptable food items, 530 out of 1,873 dairy samples, which corresponds to 28.3% of dairy products (Kharroubi et al., 2020). Furthermore, of the unacceptable dairy products 19.7% were contaminated with levels of *E. coli* exceeding the

maximum acceptable limit and 15.7% of these unacceptable dairy products were contaminated with *Staphylococcus aureus.* In addition, some cheese samples were found to harbor unacceptable levels of potentially harmful foodborne pathogens, including *Salmonella* (1.6%), *Listeria monocytogenes* (23.7%), and *Clostridium botulinum* (4.5%) (Kharroubi et al., 2020). This is problematic since dairy is a major source of nutrition amongst the Lebanese population. Furthermore, amongst the tested foodborne pathogenic agents, there was no testing for *Campylobacter jejuni,* which are the leading causes of bacterial gastroenteritis. Furthermore, the microbiological testing, conducted by the campaign, was not comprehensive as it did not include viral foodborne pathogens such as norovirus, the leading cause of foodborne gastroenteritis, and hepatitis A, which resulted in 10,400 cases between 2005 and 2017 (Kharroubi et al., 2020). But also, no testing for parasitic foodborne pathogens, such as *Cryptosporidium,* that is recently contributing to infections, was performed (Kharroubi et al., 2020). No testing for the presence of potential chemical contaminants was performed either (Kharroubi et al., 2020). Furthermore, the food safety campaign launched by the Lebanese Ministry of Public Health was suspended in 2017. In fact, the last weekly report published on the Ministry of Public Health's website covered the period spanning from 29/12/2016 to 05/01/2017 (Ministry of Public Health (MoPH). Since then, no updates to the "Food Safety" section of the ministry's website have been made. Furthermore, food safety knowledge remains basic amongst employees of various sectors amongst which the health care sector. A study by Bou-Mitri et al., assessed the knowledge, attitude, and practices of food handlers in Lebanese hospitals. 254 food handlers were recruited for the study (2018). Participants demonstrated general knowledge regarding food safety. The respondents were knowledgeable regarding certain aspects related to the

advanced preparation of food and the reheating of food and its contribution to food poisoning with respective percentages of 58.7% and 78.3% (Bou-Mitri et al., 2018). In addition, 91.7% of participants knew that the inappropriate use of cleaning and sanitization agents can increase the risk of food poisoning (Bou-Mitri et al., 2018). However, the knowledge of surveyed food handlers remained vague and basic. In fact. According to Bou-Mitri et al., the food handlers, despite the majority having received food safety trainings, exhibited very poor knowledge about the type of foods associated with some foodborne diseases (2018). This could be related to the educational background of the handlers or to drawbacks in the training programs in terms of scientific terms (Bou-Mitri et al., 2018). As of the attitudes there was a misconception amongst the food handlers regarding the prevention of food contamination in that about 60.6% of them believed that wearing protective clothing, including cap, masks, protective gloves, and other types of clothing are enough to prevent food contamination (Bou-Mitri et al., 2018). The main problems when it comes to the practices of food handlers in Lebanese hospitals that were revealed in this study were mainly the use of the same utensils to handle raw food to handle cooked foods, handling foods with untreated lesions covering the hands, and thawing of food at room temperature for 22.4%, 37.8%, and 72.8% of food handlers in the Lebanese hospitals (Bou-Mitri et al., 2018). The findings by Bou-Mitri et al. reveal a major problem related to food safety in Lebanon. Indeed, the fact that food handlers at the heart the Lebanese healthcare sector exhibit flaws in food safety knowledge, attitudes, and practices can be indicative of a nationwide multi-sectoral food safety problem.

Another major problem in Lebanon that ties-in with food safety in Lebanon which is antibiotic resistance. Lebanon suffers from high toll of antibiotic resistance that is partly due to antibiotic misuse. Limited and basic knowledge about antibiotics amongst the Lebanese

population is a leading cause of antibiotic misuse. A form of antibiotic misuse is self-medication with antibiotics to treat non-bacterial infections and any related symptoms. In a study by Jamhour et al., it was found that 61% of surveyed participants confirmed the use antibiotics to treat a common cold (2017). There was a high correlation between self-medication with antibiotics and the educational level (Jamhour et al., 2017). This draws a relationship between the education and knowledge about antibiotics use and the practices by users. However, the misuse of antibiotics spans beyond just lack of knowledge or basic knowledge about antibiotics and takes the form of habit. In fact, according to Jamhour et al., 83% of participants knew that the misuse of antibiotics could result in antibiotic resistance (2017). Furthermore, according to Alhomoud et al., the two main forms of self-medication with antibiotics are taking antibiotics without prescription and borrowing antibiotics from friends or relatives (2017). Alhomoud et al., found the prevalence of self-medication with antibiotics to range from 19% to 82% depending on various factors such as age, sex, education, and income levels (2017). Another major problem is the use of critically important antibiotics, that are designed to treat severe bacterial infections, in various other sector such the agricultural and farming sectors. An example of critically important antibiotic that is being used in the agricultural and farming practices is colistin. Colistin is a drug of the Polymyxin class and is a last resort antibiotic to treat multidrug-resistant bacterial infections. Colistin is classified as “Highest Priority” use by the World Health Organization (WHO). A study by Kassem et al. revealed that twelve brands of antibiotics, used in animal farming to treat or in certain cases prevent infections, contained colistin (2019). Even though the labels of these antibiotics do not mention their use as growth promoters within their instruction, there were instances where it was heard that these antibiotics are were being sold for that

purpose (Kassem et al., 2019). Furthermore, it is important to note that most of these colistin-containing antibiotics are used in poultry production. In fact, all the twelve brands of antibiotics target poultry (Kassem et al., 2019). The unregulated and excessive use of this highly important antibiotic in the farming practices has contributed to the increase of the selective pressure of colistin resistant bacteria. Furthermore, a study conducted by Kassaify et al. on main dairy farms in Lebanon revealed that the aminoglycosides gentamicin and streptomycin are the most frequently used antibiotics in the farming sector (2013). Both these antibiotics are used in human therapeutic treatments and being of the aminoglycosides class they are classified by the WHO as "High Priority" (Kassaify et al., 2013; World Health Organization [WHO], 2016). Furthermore, according to Kassaify et al., Despite the interviewed farmers' claims of using antibiotics in accordance with international regulations and the prescriptions of the antibiotics, still antibiotic residues were detected in collected milk samples (2013). Apart from affecting the gut microbiota, the presence of antibiotic residues could be indicative of an excessive and extensive use of gentamicin and streptomycin in the farming practices (Kassaify et al., 2013). The potential excessive and prolonged use of these antibiotics, in themselves, could indicate a potential development of antimicrobial resistance against them. This was validated by the findings by Kassaify et al., 2013. In fact, out of a total of 21 *Staphylococcus aureus* isolates 100% and 95% were resistant to gentamicin and streptomycin, respectively. But also, out of a total of 10 *E. coli* isolates 100% and 60% were found to be resistant to gentamicin and streptomycin, respectively. And thus, raw milk which is the basis of cheese and other dairy products is contaminated with antibiotic resistant bacteria. This is problematic especially that some of the locally produced cheeses are raw. Furthermore, a study by Dankar et al. assessed the

knowledge, attitudes, and perceptions of Lebanese dairy farmers regarding the use of antibiotics in the farming sector. There was misconception in some of the farmers' knowledge regarding the reasons for the use of antibiotics in the farming sector. In fact, despite a majority that agreed that antibiotics should only be administered to treat animal infections, some farmers stated that antibiotics can also used for prophylaxis and growth promotion (Dankar et al., 2022). Furthermore, when asked to elaborate on antibiotic misuse, most farmers stated that antibiotics are deemed misused only when they are administered in overdose to animals (Dankar et al., 2022). These farmers believed that an overdose administration to animals will impact humans by their consumption of animal meat and milk that is contaminated with antibiotic residues (Dankar et al., 2022). Only few farmers stated that antibiotic misuse consists to the administration in underdose to animals in addition to its administration in overdose to animals (Dankar et al., 2022). This shows contrasting knowledge around the use of antibiotics in farming settings. But also, the farmers that were interviewed in the study didn't mention that part of antibiotic misuse consisted also in the use of critically important antibiotics, made to treat severe human bacterial infections in a healthcare setting, in the animal and farming sector. In addition, when asked about the benefits of reducing antimicrobial use (AMU), most farmers answered that the major benefit would be a reduction in antimicrobial resistance (AMR) (Dankar et al., 2022). Some of the interviewed farmers, however, considered the major advantages in the reduction of AMU are economic. In fact, these advantages included preserving the image of the dairy industry, improving animal health, and reducing the withhold of animals' milk and meat (Dankar et al., 2022). As for the disadvantages in the reduction of AMU, most farmers agreed that the use of antibiotics in themselves did not pose any adverse health risks, but rather stopping the

administration of antibiotics to animals (Dankar et al., 2022). They believed that animal illness and eventual death and the economic loss that results are a worse outcome of cutting off antibiotics in their farming practices than AMR (Dankar et al., 2022).

Another major problem that ties-in to antibiotic resistance is the environmental pollution in Lebanon. In fact, the high levels of fecal pollution in Lebanese natural water sources contributes to the circulation of antibiotic resistance genes in different food systems. In a study by Diab et al., it was shown that Lebanese natural water sources are contaminated with antibiotic resistant *Enterobacteriaceae* harboring extended spectrum β-lactamase (ESBL) and carbapenemase-encoding genes (2018). This is problematic because infections by ESBL-producing *Enterobacteriaceae* are hard to treat as antibiotics available are not effective in treating these infections. These types of infections often require intravenous (IV) injections with carbapenems, as they are amongst the few antibiotics left that can treat them (Centers of Disease Control and Prevention [CDC]). In addition, infections by carbapenemase-producing *Enterobacteriaceae* are also problematic because, carbapenemases have versatile hydrolytic capacities that can even hydrolyze carbapenems, which are considered in a way as last resort antibiotics (Centers of Disease Control and Prevention [CDC]). This makes the treatment of infections by ESBL-producing *Enterobacteriaceae* even harder. The types of water sources contaminated include estuary, spring, and well water sources. contamination of these natural water sources occurs by the release of untreated household, farm, and hospital effluent (Diab et al., 2018). For instance, well water in Lebanon is heavily polluted, especially on the coastal region, due to sea water infiltration. In fact, the groundwater is infiltrated by seawater at its highest, at the level of Beirut (Saadeh and Wakim, 2017). this infiltration of seawater into groundwater is a potential

contributor of the dissemination of antibiotic resistance genes into domestic water sources. This is problematic since spring and well water sources are used for direct consumption and used in washing ready to eat fruits and vegetables or to prepare food (Diab et al., 2018). As for estuary it is used for animal consumption and farming practices (Diab et al., 2018). According to Diab et al., The tested estuary water samples were found to harbor CTX-M-15 producing *K. pneumoniae* (3 isolates) and CTX-M-15, CTX-M-55, CMY-42, and SHV-12-producing *E. coli* (12 isolates). While *E. coli* can be non-pathogenic, the extraintestinal pathogenic strain *E. coli* (B2) of sequence ST121 was detected in the estuary water samples as well as in spring water samples. This is highly problematic because this *E. coli* clone is considered to be highly pathogenic due to the wide spectrum of infections that it causes in both the community and the clinical setting and due to the high number of virulence genes that it contains (Nicolas-Chanoine et al., 2014). The presence of *K. pneumoniae* in the estuary water samples further confirms contamination by hospital effluent since, species of *Klebsiella*, with the most notable being *K. pneumoniae*, are associated with infections in the healthcare settings (Centers of Disease Control and Prevention [CDC]). This is also problematic since the farming environment can affect the microbiological quality of milk (Kassaify et al., 2013). And thus, the consumption of contaminated estuary can lead to the contamination of raw animal milk with antibiotic resistant pathogens. This is evident in the study by Diab et al., which detected the presence of ESBL and carbapenemase harboring *Enterobacteriaceae* in raw bovine milk (2017). In fact, CTX-M-15 producing *K. pneumoniae* were detected in the raw bovine milk with a 30.2% prevalence, deemed high. But also, 1 *K. pneumoniae* isolate and 7 *E. coli* isolates, sharing the sequence type ST530, were found to

produce the carbapenemase OXA-48 (Diab et al., 2017). Water pollution is also problematic as it can affect the hygiene of the workers in local dairy production facilities.

On the other hand, with the prevalence of small-scale local dairy production facilities in Lebanon, the problem of potential contamination, with foodborne pathogens, during different stages arises. One of the foodborne pathogens that could result in the contamination of dairy production is *Staphylococcus aureus. S. aureus* is an opportunistic gram -positive pathogenic bacterium that colonizes the skin and nose of 25% of humans as well as animals (Centers of Disease Control and Prevention [CDC]). It is a toxigenic bacterium capable of inducing food poisoning in humans by their consumption of food containing the bacterial enterotoxin (Centers of Disease Control and Prevention [CDC]). A In addition, *S. aureus* is a primary causative agent of contagious mastitis in cows (Kümmel et al., 2016). Mastitis-causing *S. aureus* has a potential to be transmitted from the cow's udder and into the dairy production. To assess the potential transmission of *S. aureus* from cattle with mastitis and into different dairy products, a study was conducted by Kümmel et al., 2016. This study was conducted on 1176 quarter milk samples from 294 cows in lactation along with their representative form as bulk tank milk samples from 18 different farms (Kümmel et al., 2016). It was found that *S. aureus* isolates from farms 3 and 13 were the strains that exhibited the most efficient entry from farm to dairy production (Kümmel et al., 2016). These isolates belong to cluster B which are characterized by the *spa* type t2953 of clonal complex 8 (CC8) that is associated with contagiousness and high prevalence within the herd (Kümmel et al., 2016). But also, strains of this cluster sequence type 8 (ST8) as well as the enterotoxin encoding genes *sea*, *sed*, and *sej* (Kümmel et al., 2016). The characteristics of cluster B strains describe genotype B of *S. aureus.* this genotype is found to be contagious (Cosandey

et al., 2016). Furthermore, according to Kümmel et al., some of cluster A dairy strains were associated with the cluster B CC8 farm strains (2016). This could suggest that the cluster B *S. aureus* adapted to the dairy production settings and developed the capacity to form biofilms on the equipment of the dairy processing plant, thus contaminating dairy products (Kümmel et al., 2016). This adaptation is suggested by the absence of capsule polysaccharide expression in cluster B and some of the cluster A *S. aureus* which in turn increases the expression of surface-associated adhesins (Kümmel et al., 2016). But also, another way *S. aureus* could be transmitted from farm to dairy is the zoonotic potential of some *S. aureus* strains. In fact, according to Kümmel et al., cluster B *S. aureus* strains of sequence type ST2945 which were the most effectively transmitted strains from farm to dairy (2016). These strains harbored genes that encode for the secretion of enterotoxins SEA, SED, and SEJ that are known for their potential toxigenic activity in humans. In addition, SEA and SED toxins are highly linked to dairy-associated foodborne outbreaks (Kümmel, 2016). Furthermore, according to Kümmel et al., the strains that were isolated were all methicillin susceptible *S. aureus* (MSSA). However, in a study by Sato et al., conducted on retail meats, milk, and cows with mastitis, the 7 *S. aureus* strains isolated from cows with mastitis were clonal complex 8 (CC8) like the *S. aureus* isolates that were classified as cluster B in the study by Kümmel et al., but different sequence type and *spa* type, were found to be MRSA (Sato et al., 2017). Furthermore, according to Sato et al., 1 of the 7 MRSA strains isolated from cows with mastitis were found to be 100% genetically identical to 3 MRSA isolates from meat (2 isolates from beef and 1 isolate from pork) and to 10 community-acquired MRSA (CA-MRSA) strains (2017). In addition, 3 MRSA strains isolated from cow with mastitis were found to be 100% identical to 2 human CA-MRSA strains (Sato et al.2017). This could be

due to the ability of MRSA to colonize both humans and cattle as part of zoonotic and reverse zoonotic transmissions. In fact, according to Schnitt and Tenhagen, most MRSA strains that colonize both humans and cattle were found to livestock-associated methicillin resistant *Staphylococcus aureus* (LA-MRSA) (2020). But also, community-associated, and healthcare-associated methicillin-resistant *Staphylococcus* aureus (CA/HA-MRSA) were isolated from cows (Schnitt and Tenhagen, 2020). For instance, WA-MRSA-1, one of the most prevalent CA-MRSA strains circulating in Australia, was isolated from a milk sample of a subclinical mastitis case (Schnitt and Tenhagen, 2020). In another instance, the human-associated epidemic Geraldine-MRSA clone (ST5, t002-I) was detected in a bovine milk sample in France (Schnitt and Tenhagen, 2020). In addition, CA-MRSA (ST72, t324-IVa) and HA-MRSA (t148-IVa) strains were detected in milk samples in Korea (Schnitt and Tenhagen, 2020). This shows that MRSA spreads vastly in the between the farm setting and the community, thus contributing to the dissemination and circulation of antibiotic resistant of *S. aureus* strains. But also, MRSA like MSSA can be transmitted from humans to the cheese via unhygienic handling.

MRSA can also cause food poisoning if it was colonizing the skin of the food handler and he touched food without prior hand washing. The severity of the food poisoning caused by MRSA, however, is same as that caused by MSSA and is independent of the antimicrobial resistance profile of *S. aureus* (Sergelidis and Angelidis, 2017). In a study by Castro et al., 162 food handlers volunteered for the assessment of their hands and nose for the presence of *S. aureus* (2016). Furthermore, if detected the *S. aureus* isolates were tested for antimicrobial susceptibility with 9 antimicrobial agents (Castro et al., 2016). But also, the strains were tested for the presence of virulence factors by SmaI-Pulse Field Gel Electrophoresis (SmaI-

PFGE). 11.1% of food handlers carried *S. aureus* on their hands, 6.2% of food handlers carried *S. aureus* on their skin and in their nose, and 4.9% of food handlers carried on hands but not in the nose (Castro et al., 2016). While food handlers with *S. aureus* on their hands and that does not originate from their nose, probably acquired it from an external source, a larger percentage of food handlers that carried *S. aureus* in their nose and on their hands, acquired these strains from their own body (Castro et al., 2016). According to Castro et al., the investigated prevalence of *S. aureus* in the nose of food handlers was found 19.8% (2016). This prevalence is included within the range of the mean nasal colonization rate in healthy adults which is (20%,30%). This mean range represents the nasal colonization rate in persistent carriers (Castro et al., 2016). Furthermore, a study by Osman et al., 5 out of 38 (13.2%) *S. aureus* strains isolated from the nasal carriages of Lebanese food handlers were methicillin-resistant *Staphylococcus aureus* (MRSA) (2019). According to Osman et al., the prevalence of MRSA in healthy carriers has heavily increased, compared to its prevalence in the last decade (2019). Furthermore, one of the isolated MRSA strains was found to be resistant to critically important antibiotics, including β-lactams, aminoglycosides, tetracycline, and fusidic acid (Osman et al., 2019). These findings shed light on the role of food handlers as healthy carriers of foodborne pathogens. This is relevant to the preparation of local artisanal cheeses in Lebanon as most of them are prepared by hand.

Lebanon is a developing country that suffers from poor infrastructures, poor wastewater management, and heavy environmental pollution. In addition, food safety in Lebanon remains basic and flawed and research on the microbiological quality of food and related foodborne illnesses remains limited. For instance, data regarding the microbiological quality of local dairy products and related research remain limited. This is the case of white

brine cheese. A particular example is Akkawi cheese. Akkawi cheese is a popular local food item that is widely consumed. However, very few research studies were conducted to assess the microbiological quality of Akkawi cheese and its role in the dissemination of antibiotic resistance. Therefore, the current study examines the microbiological quality of Akkawi cheese samples collected from different retail stores in Beirut. Fecal coliforms and *Escherichia coli* are used as indicators of fecal pollution and *Staphylococcus aureus* is used as hygiene indicator. Furthermore, the antimicrobial resistance status of *E. coli* and *S. aureus* is assessed.

CHAPTER II

MATERIALS AND METHODS

A. Sample Collection

Fifty samples of Akkawi cheese were collected from different retail stores in Beirut. The Akkawi cheese tested included Akkawi Baladi and Akkawi Cheeky. The sample analysis was conducted on the same day the sample was collected. In rare instances, the sample was collected a day prior to the time of analysis in which case the cheese was stored in the fridge at a temperature of 4ºC. The sample analysis took place at the microbiology lab in the Nutrition and Food Sciences (NFSC) department of the Faculty of Agriculture and Food Sciences (FAFS) at the American University of Beirut (AUB), Bliss Street, Hamra, Beirut, Lebanon. The cheese samples were cut from the large cheese molds and were placed in sterile sampling cups. Sampling was performed under aseptic conditions. Brine, if available, was collected in sterile sample cups and was analyzed along with the cheese sample.

B. Culture and Isolation of Escherichia coli and Staphylococcus aureus

25 grams portions of the samples were homogenized with 225 ml of buffered peptone water (BPW) using a stomacher. Two serial 10-fold dilutions in BPW were conducted using the homogenized mixture as well as the brine. The 10^{-1} as well as the 10^{-3} dilutions of the cheese-peptone mixture and the 10^{0} as well as the 10^{-2} dilutions of the brine were plated on selective media. The plating procedure consisted in plating 100µl of each dilution for both the cheese sample as well as the brine sample on both types of selective media plates. Chromogenic Tryptone Bile X-glucuronic (TBX) agar used to isolate *E. coli.* TBX agar plates

were incubated at a temperature of 37ºC for 24 h. Baird-Parker agar (BPA) was used to isolate *Staphylococcus aureus.* BPA plates were incubated at a temperature of 37ºC for 48 h. Glycerol stocks of the cheese and brine samples were prepared by adding 20 ml of 80% glycerol solution to 40 ml of the cheese-peptone mixture and 5 ml of 80% glycerol solution to 10 ml of brine solution and storing them in the freezer at a temperature of -20ºC. Following, incubation the number of *E. coli,* fecal coliforms, and *S. aureus* were counted. Pure *E. coli* and *S. aureus* colonies that were isolated, were subjected to heavy streaking on the appropriate agar media and incubated at 37ºC for 24 h. As for impure colonies that were isolated, they were purified by successive streaking on the corresponding agar media and then subjected to heavy streaking and incubation at 37ºC for 24 h. after pure colonies grew on heavily streaked plates, they are isolated and used to prepare glycerol stocks. *E. coli* glycerol stocks were prepared by dissolving the bacterial colonies in 1000µl of LB broth and adding 500µl of 80% glycerol solution. As for *S. aureus* glycerol stocks, they were prepared by dissolving the bacterial colonies in 1000µl of brain-heart infusion (BHI) broth and adding 500µl of 80% glycerol solution. The glycerol stocks are then stored in the freezer at -80ºC, until the antimicrobial resistance (AMR) testing started.

C. Antimicrobial Resistance (AMR)

Antibiotic resistance test of the isolated strains was done using the disk diffusion method. At first the *E. coli* were cultured on TBX agar plates, 24 hours prior to conducting the AMR test. few colonies of each isolate were taken using an inoculating loop and re-dissolved in Mueller-Hinton (MH) broth. The optical density, at a wavelength of 600 nm (OD_{600}) of the MH broth, was adjusted to 0.050, using a spectrophotometer. uninoculated

MH broth was used as blank. Once the optical density was adjusted, swabbing of the inoculated MH broth on Mueller-Hinton (MH) agar was performed. Subsequently, antibiotic disks were fixed on the plates, using a sterile tonsil. The antimicrobial disks used in AMR testing include penicillin (PEN), ampicillin (AMP), amoxicillin-clavulanate (AMC), cefepime (FEP), cefotaxime (CTX), cephalexin (LEX), cefixime (CFM), doripenem (DOR), imipenem (IPM), meropenem (MEM), gentamicin (GEN), kanamycin (KAN), streptomycin (STR), tetracycline (TET), ciprofloxacin (CIP), norfloxacin (NOR), trimethoprim-sulfamethoxazole (SXT), chloramphenicol (CHL), and colistin (CS). Erythromycin (ERY) was used as control. This was followed by a 24-hour incubation of the plates at a temperature of 37 ºC. Following the incubation, the strains were determined as resistant, intermediate, or susceptible to the antibiotic, based on the measured diameter of the zone of inhibition that was formed around the antibiotic disk.

D. Measurement of pH and Salinity of Brine Samples

The pH of the brine is measured using the pH-meter. The electrode of the pH-meter was placed in the brine sample and pH value is determined.

The electric conductivity of the cheese brine samples was measured using the conductometer. The electrode of the conductometer was placed in the brine sample and electric conductivity value is determined. The salinity of the brine is derived from the conductivity, using the formula:

Salinity (g/L) = $(\text{Conductivity (mS/cm)})^{1.0878} * 0.4665$

E. Polymerase Chain Reaction (PCR)

The primer working solutions used in the PCR reactions were obtained by performing a 1:5 (primer: DNase free water) dilution of the primer mother solution.

1. For Escherichia coli

Boil prep for *E. coli* isolates was conducted by suspending the isolates in 100µl DNase free water. The mixture was then boiled at 95ºC for 12 minutes using a VWR® thermal cycler. This was followed by the centrifugation of the Boil prep mixture at 13.3 rpm for 2 minutes. The supernatant was then extracted.

The PCR reactions for *E. coli* consisted of 6µl DNase free water, 10µl of (REDTaq® ReadyMix™ PCR Reaction Mix 2x concentrate), 0.5µl the primers, and 3µl of the DNA to be analyzed. The total volume of each PCR reaction was 20µl. The PCR reactions were conducted using a Bio-Rad® C1000 thermal cycler.

16S rRNA PCR was conducted as a solid confirmation of *E. coli* isolates. The targeted gene was the 16S *E. coli* gene and the primers 16S-1-f (AAGAAGCTTGCTTCTTTGCTGAC) and 16S-1-r (AGCCCGGGGATTTCACATCTGACTTA). The PCR conditions consisted of an initial denaturation at 95°C for 2 minutes, followed by 38 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 45 seconds, and extension 72°C for 1 minute followed by a final extension at 72°C for 10 minutes.

2. For Staphylococcus aureus

Boil prep for *S. aureus* isolates was conducted by suspending the isolates in a mixture of 5µl of proteinase K and 100µl of Triton X buffer (100 mM NaCl, 10 mM EDTA, 1 mM Tris HCl, 1% Triton X-100). The mixture was then incubated at 37ºC for 10 minutes before being boiled at 95ºC for 12 minutes using a VWR® thermal cycler. This was followed by the centrifugation of the Boil prep mixture at 13.3 rpm for 2 minutes. The supernatant was then extracted.

The PCR reactions for *S. aureus* consisted of 6µl DNase free water, 10µl of (REDTaq® ReadyMix™ PCR Reaction Mix 2x concentrate), 0.5µl the primers, and 3µl of the DNA to be analyzed. The total volume of each PCR reaction was 20µl. The PCR reactions were conducted using a Bio-Rad® C1000 thermal cycler.

*fem*B gene detection was conducted using PCR to confirm *S. aureus* isolates that exhibited the morphological criteria typical to *S. aureus.* the forward primer is (TTACAGAGTTAACTGTTACC) and the reverse primer is (ATACAAATCCAGCACGCTCT). The PCR conditions consisted of an initial denaturation at 95°C for 4 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 50°C for 45 seconds, and extension 72°C for 3 minutes followed by a final extension at 72°C for 10 minutes.

*mec*A gene detection was conducted using PCR to detect methicillin resistant *S. aureus* (MRSA) isolates that exhibited the morphological criteria typical to *S. aureus.* the forward primer is (GTAGAAATGACTGAACGTCCGATAA) and the reverse primer is (CCAATTCCACATTGTTTCGGTCTAA). The PCR conditions consisted of an initial denaturation at 95°C for 4 minutes, followed by 30 cycles of denaturation at 94°C for 1

minute, annealing at 50°C for 45 seconds, and extension 72°C for 3 minutes followed by a final extension at 72°C for 10 minutes.

F. Statistical Analysis

A simple linear regression analysis was conducted using SPSS 23.0, to study the correlation between the bacterial densities in the Akkawi cheese samples and the bacterial densities in the brine samples. But also, simple linear regression analysis to study the correlation between the bacterial densities and the parameters of the brine: pH and salinity (g/L).

CHAPTER III

RESULTS

A. Bacterial Densities

1. Bacterial Densities in Cheese Samples

Results revealed major fecal pollution of cheese samples. In fact, of a total of 50 Akkawi cheese samples, 40 (80%) cheese samples were contaminated with *Escherischia coli. E. coli* level ranged from about 2.92 to 7.31 log_{10}CFU/g heavily exceeding the maximum acceptable level of the Lebanese standard LIBNOR **(Figure 1)**. In fact, out of the the 40 Akkawi cheese samples contaminated with *E. coli*, 37 samples (92.5%) exceeded the maximum acceptable level of 3 log_{10}CFU/g (1000 CFU/g) as per the 2002 LIBNOR standard for Akkawi cheeses (2002:223). Furthermore, 40 out of 40 (100%) of contaminated with *E. coli,* were found to exceed the maximum acceptable level of <1 log_{10}CFU/g (<10 CFU/g) as per the LIBNOR 2003 standard for Akkawi cheeses (2003:495) **(Figure 1)**. Likewise, fecal coliform densities were relatively high, ranging from about 2.92 to 7.88 log_{10}CFU/g. Despite the absence of a Lebanese standard for fecal coliforms in Akkawi Cheese, these densities were deemed extremely high **(Figure 1)**. 43 out of 50 (86%) cheese samples were contaminated with fecal coliforms. Despite only 16 out of 50 (32%) of Akkawi cheese samples were contaminated with *Staphylococcus aureus*, the densities exceeded the maximum acceptable levels based on Lebanese standards **(Figure 2)**. In fact, 14 out of 16 (87.5%) of contaminated samples exhibited *S. aureus* densities that exceeded the maximum acceptable level of 3 log_{10}CFU/g (1000 CFU/g) as per the 2002 LIBNOR standard for Akkawi cheese (2002:223). Furthermore, 16 out of 16 (100%) of the contaminated samples

exhibited *S. aureus* densities that exceeded the maximum acceptable level of 2 $\log_{10}$CFU/g (100 CFU/g) as per the 2003 LIBNOR standard for Akkawi cheese (2003:495) **(Figure 2)**.

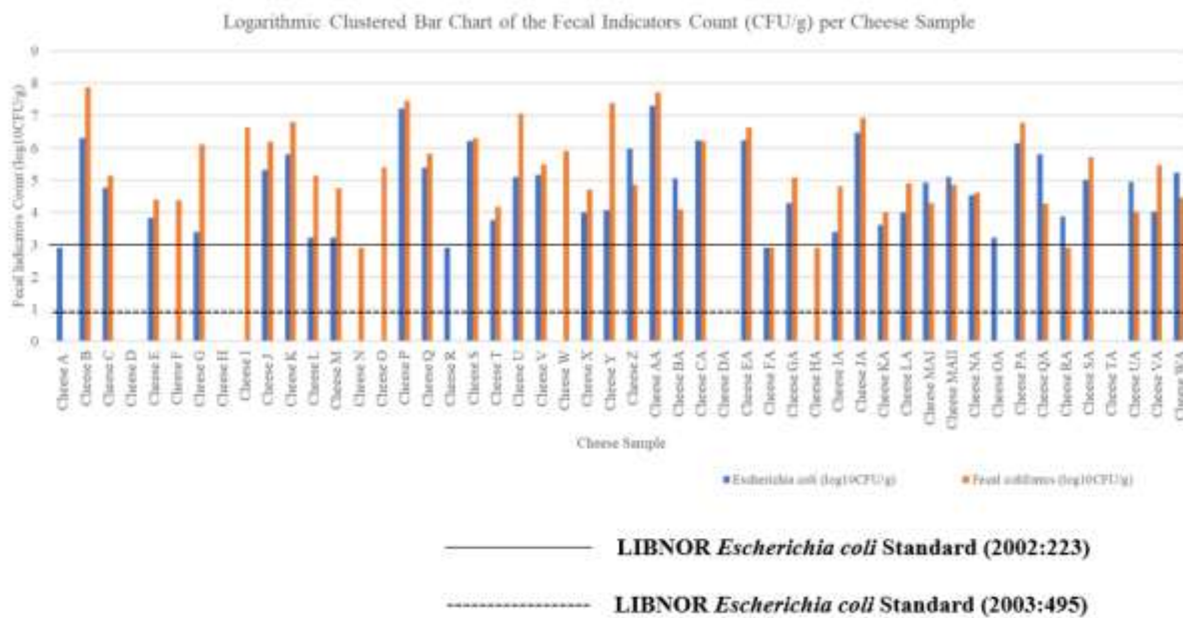


Figure 1. *Escherichia coli* and fecal coliform densities ($\log_{10}$CFU/g) in Akkawi cheese samples

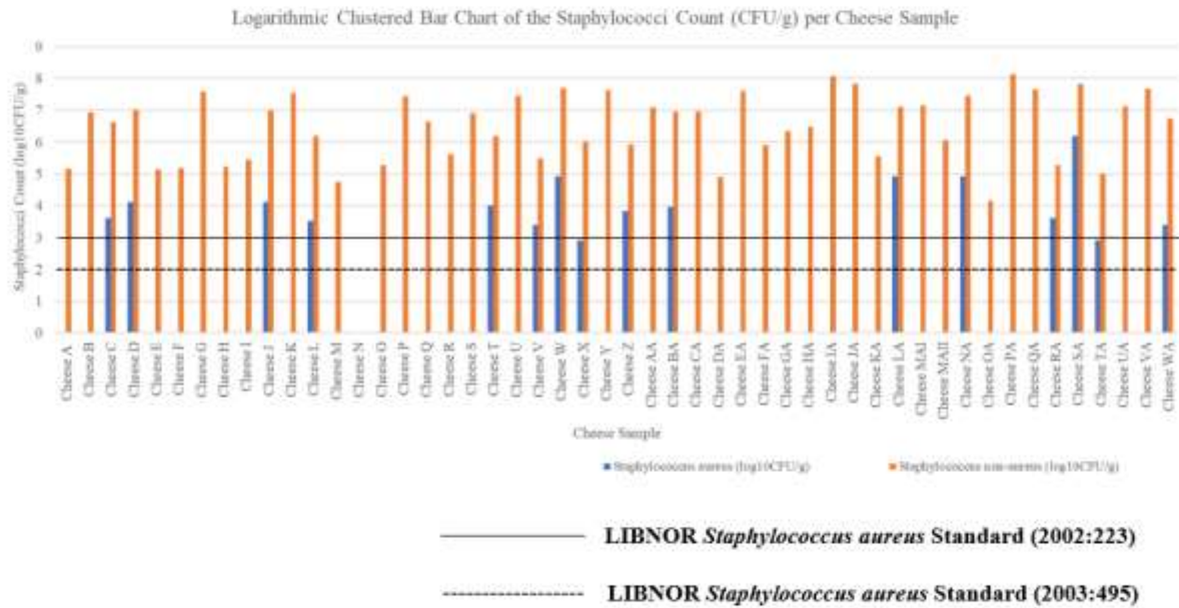


Figure 2. *Staphylococcus aureus* and other staphylococci densities (log_{10}CFU/g) in Akkawi cheese samples

2. Bacterial Densities in Brine Samples

13 brine samples were collected. Out of the 13 brine samples, 4 (30.8%) were contaminated with *E. coli*, 10 (77.0%) were contaminated with fecal coliforms **(Figure 3)**, and 1 (7.7%) was contaminated with *S. aureus* **(Figure 4)**. Despite the absence of a Lebanese standard for the microbiological quality white cheese brine, the densities were relatively high. Indeed, 100% of samples contaminated with *E.* coli, and 90% of samples contaminated with fecal coliforms exhibited densities that exceeded 3 log_{10}CFU/ml **(Figure 3)**. As for the *Staphylococcus aureus* the comntaminated sample exhibited a count of 3 log_{10}CFU/ml **(Figure 4)**.

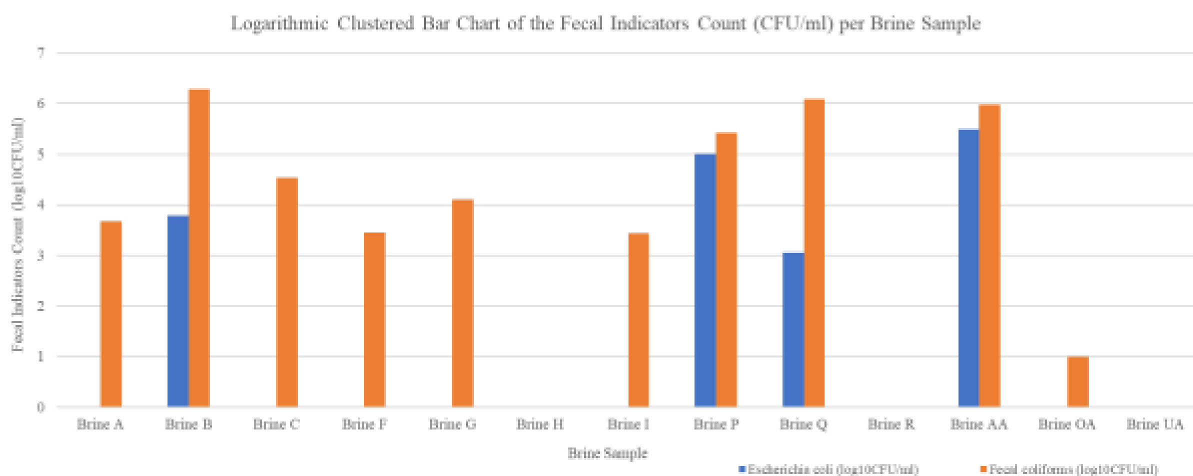


Figure 3. *Escherichia coli* and fecal coliform densities (log_{10}CFU/ml) in Akkawi brine samples

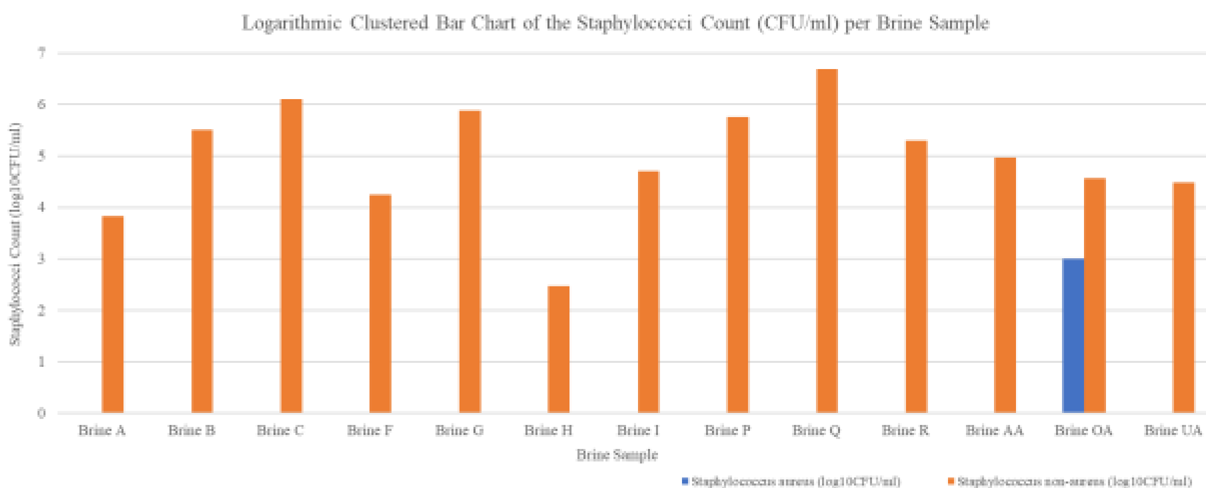


Figure 4. *Staphylococcus aureus* and other staphylococci densities (log_{10}CFU/ml) in Akkawi brine samples

	Cheese *E. coli*	Cheese fecal coliforms	pH	Salinity (g/L)
Brine *E. coli*	0.923	-	0.017	0.265
Brine fecal coliforms	-	0.800	0.232	0.359
Cheese *E. coli*	-	-	0.077	0.309
Cheese fecal coliforms	-	-	0.382	0.290

Table 1. Correlation coefficients (R) from Pearson correlation analysis between the brine *Escherichia coli* and fecal coliforms densities and the cheese *Escherichia coli* and fecal coliforms densities, as well as the pH, and salinity (g/L) of the brine and from Pearson correlation analysis between the cheese *Escherichia coli* and fecal coliforms densities and the pH and salinity (g/L) of the brine.

	Cheese *S. aureus*	Cheese other staphylococci	pH	Salinity (g/L)
Brine *S. aureus*	0.091	-	0.022	0.058
Brine other staphylococci	-	0.006	0.402	0.278
Cheese *S. aureus*	-	-	0.323	0.165
Cheese other staphylococci	-	-	0.145	0.418

Table 2. Correlation coefficients (R) from Pearson correlation analysis between the brine *Staphylococcus aureus* and other staphylococci densities and the cheese *Staphylococcus aureus* and other staphylococci densities, pH, and salinity (g/L) and from Pearson correlation analysis between the cheese *Staphylococcus aureus* and other staphylococci densities and the pH and salinity (g/L) of the brine.

A strong positive linear association was found between the densities of *E. coli* and fecal coliform strains isolated from the cheese and the densities of isolates from the brine, with respective Pearson correlation coefficients of R=0.923 and R=0.800 (**Table 1**). Conversely, a negligeable positive linear association was found between the densities of *S. aureus* and other staphylococci strains isolated from the cheese and the densities of isolates from the brine, with respective Pearson correlation coefficients of R=0.923 and R=0.800 (**Table 2**). As such, the brine samples can only be used as a predictor for *E. coli* and fecal coliform densities.

While a negligeable positive linear association was found between the *S. aureus* densities isolated from the brine and both the pH and the salinity of the brine solution with respective Pearson correlation coefficients R=0.022 and R=0.058, a negligeable positive linear association was only found between the *E. coli* densities isolated from the brine and the pH of the brine solution (R=0.017) **(Table 1).** Whereas a weak to moderate positive linear association between the *E. coli* densities isolated from the brine and the salinity of the brine solution (R=0.265) **(Table 1).** A moderate positive linear association was found between the fecal coliform densities isolated from the brine and the salinity of the brine solution (R=0.359) and between other staphylococci densities isolated from the brine and the pH of the brine solution (R=0.402) **(Table 2).** A weak positive linear association was found between the fecal coliform densities isolated from the brine and the pH of the brine (R=0.232) **(Table 1).** A weak to moderate positive linear association was found between other staphylococci densities and the salinity of the brine solution (R=0.278) **(Table 2).**

While a negligeable positive linear association was found between the *E. coli* densities isolated from the cheese and the pH of the brine (R=0.077), a moderate positive

linear association was found between the fecal coliform densities isolated from the cheese and the pH of the brine (R=0.382) **(Table 1).** A moderate positive linear association was found between the *E. coli* densities isolated from the cheese and the salinity of the brine (R=0.309), whereas a weak to moderate positive linear association was found between the fecal coliform densities isolated from the cheese and the salinity of the brine (R=0.077) **(Table 1).** As for *Staphylococcus*, a moderate positive linear association was found between the *S. aureus* densities isolated from the cheese and the pH of the brine (R=0.323) **(Table 2).** Whereas a weak positive linear association was found between other staphylococci densities isolated from the cheese and the pH of the brine (R=0.145) **(Table 2).** Conversely, a weak positive linear association was found between the *S. aureus* densities isolated from the cheese and the salinity of the brine (R=0.165), while a moderate positive linear association was found between other staphylococci densities isolated from the cheese and the salinity of the brine (R=0.165) **(Table 2).**

B. Antimicrobial Resistance of *Escherichia coli* Isolates

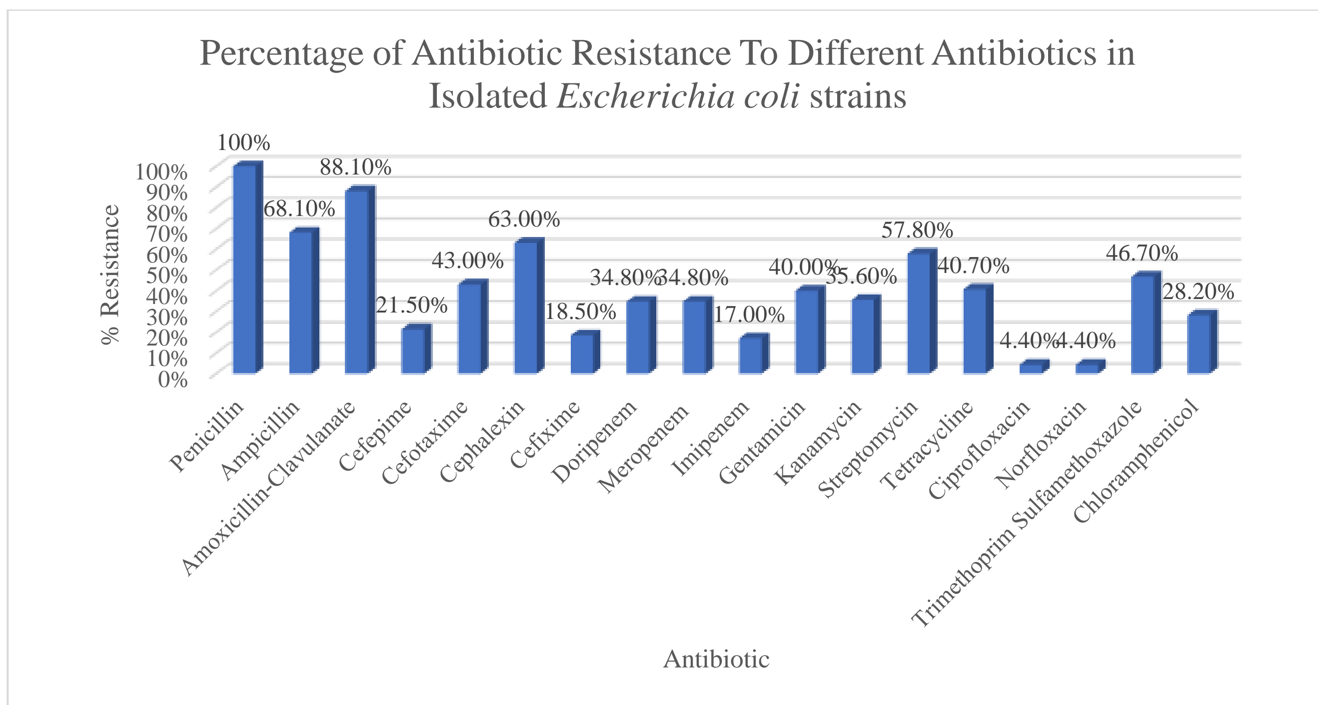


Figure 5. Percentage of antimicrobial resistance to different antibiotics amongst *Escherichia coli* isolated from Akkawi cheese and the collected brine.

Antibiotic	Akkawi Cheese Isolates n=118 (%)	Akkawi Brine Isolates n=17 (%)	Total n=135 (%)
Penicillin	118 (100)	17 (100)	135 (100)
Ampicillin	80 (67.8)	12 (70.6)	92 (68.1)
Amoxicillin-Clavulanate	104 (88.1)	15 (88.2)	119 (88.1)
Cefepime	24 (20.3)	5 (29.4)	29 (21.5)
Cefotaxime	48 (40.7)	10 (58.8)	58 (43.0)
Cephalexin	78 (66.1)	7 (41.2)	85 (63.0)
Cefixime	21 (17.8)	4 (23.5)	25 (18.5)
Doripenem	45 (38.1)	2 (11.8)	47 (34.8)
Meropenem	44 (37.3)	3 (17.6)	47 (34.8)
Imipenem	21 (17.8)	2 (11.8)	23 (17.0)
Gentamicin	48 (40.7)	6 (35.3)	54 (40.0)
Kanamycin	42 (35.6)	6 (35.3)	48 (35.6)
Streptomycin	68 (57.6)	10 (58.8)	78 (57.8)
Tetracycline	47 (39.8)	8 (47.1)	55 (40.7)
Ciprofloxacin	6 (5.1)	-	6 (4.4)

Norfloxacin	5 (4.2)	1 (5.9)	6 (4.4)
Trimethoprim-Sulfamethoxazole	55 (46.6)	8 (47.1)	63 (46.7)
Chloramphenicol	32 (27.1)	6 (35.3)	38 (28.1)
Multidrug Resistant Isolates	**90 (76.3)**	**11 (64.7)**	**101 (74.8)**

Table 3. Prevalence of phenotypical antimicrobial resistance in *Escherichia coli* isolated from Akkawi cheese and the collected brine.

The Kirby-Bauer disk diffusion assay was conducted to screen a total of 135 *Escherichia coli* isolates, 118 strains were isolated from cheese samples and 17 were isolated from the collected brine samples. These *E. coli* isolates were confirmed via 16S-rRNA PCR testing. Overall, the highest observed antibiotic resistances amongst the *E. coli* isolates were against amoxicillin-clavulanate, ampicillin, cephalexin, and streptomycin, with respective rates of about 88.1%, 68.1%, 63.0%, and 57.8% **(Figure 5)**. The lowest observed resistance was the fluoroquinolones ciprofloxacin and norfloxacin with a resistance rate of 4.4% **(Figure 5)**. A similar resistance pattern was observed for the Akkawi cheese *E. coli* strains. In fact, the highest observed antibiotic resistances were against amoxicillin-clavulanate, ampicillin, cephalexin, and streptomycin with respective rates of about 88.1%, 67.8%, 66.1%, and 57.6% **(Table 3).** As for the *E. coli* strains isolated from the brine samples, they also exhibited a similar pattern of resistance as those isolated from cheese samples in that highest resistance was observed against amoxicillin-clavulanate (88.2%), ampicillin (70.6%), and Streptomycin (58.8%), however, resistance against the cephalosporin cefotaxime (58.8%) was higher than that against cephalexin (41.2%) **(Table 3)**. Resistance against fluoroquinolones was the lowest amongst both cheese and brine isolated *E. coli*

strains. Indeed, the respective resistance rates for ciprofloxacin and norfloxacin were 5.1% and 4.2% **(Table 3).** As for the brine isolates a low resistance resistance against Norfloxacin (5.9%) was observed and no phenotypic resistance against ciprofloxacin was observed **(Table 3).** Of the 135 *E. coli* isolates a total of 101 strains (74.8%) were found to be multidrug resistant (MDR) as they were resistant to at least three classes of antibiotics **(Table 3).** The proportion of MDR strains was found to be higher amongst the *E. coli* strains isolated from cheese than the strains isolated from the brine with a percentage of 76.3% against 64.7% **(Table 3).**

Sample	**Number of antibiotics**	**Resistance profile**	**Number of isolates**	**Isolates**
Akkawi Cheese	1	PEN	8	“Cheese B-Dilution 10^-1 (1) [RE]”, “Cheese V-Dilution 10^-1 (1) [RE]”, “Cheese W-Dilution 10^-1 (2) [RE]”, “Cheese X-Dilution 10^-1 (1)”, “Cheese X-Dilution 10^-1 (2) [RE]”, “Cheese Y-Dilution 10^-1 (2) [RE]”, “Cheese BA-Dilution 10^-1 (1) [RE]”,
	2	PEN-AMC	4	“Cheese T-Dilution 10^-1 (3) [RE]”, “Cheese Y-Dilution 10^-3(2)”, “Cheese Y-Dilution 10^-1 (1) [RE]”, “Cheese RA-Dilution 10^-1 (1)”
	3	PEN-AMC-CTX	2	“Cheese T-Dilution 10^-1 (1) [RE]”, “Cheese Y-Dilution 10^-1 (3) [RE]”
	3	PEN-AMP-SXT	2	“Cheese A-Dilution 10^-1-Trial”, “Cheese T-Dilution 10^-1 (4) [RE]”
	4	PEN-AMP-AMC-LEX	2	“Cheese X-Dilution 10^-3”, “Cheese HA-Dilution 10^-1 (2) [RE]”

	5	PEN-AMP-AMC-LEX-IPM	2	"Cheese GA-Dilution 10^-1 (1)", "Cheese GA-Dilution 10^-1 (2)"
	6	PEN-AMP-AMC-LEX-DOR-MEM	2	"Cheese U-Dilution 10^-1 (2)", "Cheese WA-Dilution 10^-1 (2)"
	7	PEN-AMP-AMC-CTX-LEX-MEM-STR	2	"Cheese X-Dilution 10^-1 (1)", "Cheese Z-Dilution 10^-3"
	8	PEN-AMP-AMC-LEX-KAN-STR-TET-SXT	3	"Cheese HA-Dilution 10^-1 (4) [RE]", "Cheese KA-Dilution 10^-1 (1)", "Cheese KA-Dilution 10^-1 (2)"
	11	PEN-AMP-AMC-LEX-DOR-GEN-KAN-STR-TET-SXT-CHL	3	"Cheese R-Dilution 10^-1 (1)", "Cheese NA-Dilution 10^-1 (2) [RE]", "Cheese VA-Dilution 10^-1 (2)"
	14	PEN-AMC-FEP-CTX-LEX-CFM-DOR-MEM-GEN-KAN-STR-TET-SXT-CHL	2	"Cheese JA-Dilution 10^-3 (1)", "Cheese JA-Dilution 10^-3 (4)"
	15	PEN-AMP-AMC-FEP-CTX-LEX-CFM-DOR-MEM-GEN-KAN-STR-TET-SXT-CHL	4	"Cheese G-Dilution 10^-1 (1)", "Cheese J-Dilution 10^-1 (4)", "Cheese JA-Dilution 10^-3 (2)", "Cheese MAII-Dilution 10^-1 (3) [RE]"
	3	PEN-AMP-STR	1	"Cheese B-Dilution 10^-1"
	12	PEN-AMP-AMC-FEP-CTX-LEX-CFM-STR-TET-CIP-NOR-SXT	1	"Cheese C-Dilution 10^-3(1)"
	8	PEN-AMP-AMC-GEN-KAN-TET-SXT-CHL	1	"Cheese C-Dilution 10^-3(2)"
	14	PEN-AMP-AMC-FEP-CTX-LEX-CFM-MEM-KAN-STR-TET-CIP-NOR-SXT	1	"Cheese C-Dilution 10^-3(3)"
	13	PEN-AMP-AMC-CTX-LEX-CFM-DOR-MEM-IPM-STR-TET-NOR-SXT	1	"Cheese C-Dilution 10^-1 (1)"
	5	PEN-AMP-AMC-LEX-DOR	1	"Cheese C-Dilution 10^-1 (3)"
	9	PEN-AMP-CTX-LEX-CFM-MEM-STR-TET-SXT	1	"Cheese C-Dilution 10^-1 (1) [RE]"
	12	PEN-AMP-AMC-LEX-GEN-KAN-STR-TET-CIP-NOR-SXT-CHL	1	"Cheese C-Dilution 10^-1 (2) [RE]"

	10	PEN-AMP-AMC-FEP-CTX-LEX-DOR-MEM-IPM-GEN	1	"Cheese E-Dilution 10^-1 (2)"
	7	PEN-AMP-AMC-CTX-DOR-MEM-STR	1	"Cheese E-Dilution 10^-1 (3)"
	11	PEN-AMP-AMC-LEX-IPM-GEN-KAN-STR-TET-SXT-CHL	1	"Cheese G-Dilution 10^-1 (3)"
	12	PEN-AMP-AMC-FEP-LEX-CFM-MEM-KAN-STR-TET-SXT-CHL	1	"Cheese J-Dilution 10^-3 (4)"
	9	PEN-AMP-AMC-IPM-GEN-KAN-STR-TET-SXT	1	"Cheese J-Dilution 10^-1 (1)"
	7	PEN-AMC-FEP-CTX-LEX-DOR-MEM	1	"Cheese J-Dilution 10^-1 (3)"
	4	PEN-AMP-GEN-STR	1	"Cheese K-Dilution 10^-1 (2) [RE]"
	3	PEN-LEX-IPM	1	"Cheese L-Dilution 10^-1 (2) [RE]"
	10	PEN-AMP-AMC-CTX-GEN-KAN-TET-CIP-SXT-CHL	1	"Cheese R-Dilution 10^-1 (2)"
	12	PEN-AMC-FEP-CTX-LEX-DOR-MEM-IPM-GEN-KAN-TET-CIP	1	"Cheese S-Dilution 10^-1 (2)"
	13	PEN-AMP-AMC-CTX-CFM-DOR-MEM-IPM-GEN-KAN-STR-TET-SXT	1	"Cheese S-Dilution 10^-1 (3)"
	7	PEN-AMP-AMC-CTX-LEX-DOR-MEM	1	"Cheese T-Dilution 10^-1 (1)"
	10	PEN-AMC-FEP-CTX-LEX-CFM-DOR-MEM-GEN-KAN	1	"Cheese T-Dilution 10^-1 (2)"
	4	PEN-AMC-CTX-DOR	1	"Cheese T-Dilution 10^-1 (2) [RE]"
	5	PEN-AMP-AMC-LEX-MEM	1	"Cheese U-Dilution 10^-1 (1)"
	4	PEN-AMC-LEX-DOR	1	"Cheese U-Dilution 10^-1 (3)"
	9	PEN-AMC-FEP-CTX-LEX-DOR-MEM-GEN-STR	1	"Cheese V-Dilution 10^-1 (1)"
	7	PEN-AMP-AMC-CTX-LEX-STR-SXT	1	"Cheese V-Dilution 10^-1 (2)"
	4	PEN-AMC-LEX-STR	1	"Cheese V-Dilution 10^-1 (3)"

	8	PEN-AMC-DOR-GEN-KAN-STR-SXT-CHL	1	“Cheese V-Dilution 10^-1 (2) [RE]”
	2	PEN-STR	1	“Cheese V-Dilution 10^-1 (3) [RE]”
	2	PEN-CTX	1	“Cheese W-Dilution 10^-1 (1) [RE]”
	6	PEN-AMC-CTX-LEX-STR-SXT	1	“Cheese X-Dilution 10^-1 (3)”
	8	PEN-AMP-AMC-FEP-CTX-LEX-STR-SXT	1	“Cheese X-Dilution 10^-1 (4)”
	6	PEN-AMC-LEX-CFM-MEM-STR	1	“Cheese Z-Dilution 10^-1 (1)”
	12	PEN-AMP-AMC-CTX-LEX-DOR-MEM-GEN-KAN-STR-TET-CHL	1	“Cheese Z-Dilution 10^-1 (2)”
	12	PEN-AMP-AMC-CTX-DOR-MEM-GEN-KAN-STR-TET-SXT-CHL	1	“Cheese Z-Dilution 10^-1 (3)”
	7	PEN-AMP-AMC-CTX-LEX-MEM-SXT	1	“Cheese AA-Dilution 10^-1 (2)”
	4	PEN-AMC-FEP-SXT	1	“Cheese AA- Dilution 10^-1”
	8	PEN-AMP-AMC-LEX-CFM-GEN-KAN-STR	1	“Cheese CA-Dilution 10^-1 (1)”
	9	PEN-AMP-AMC-CTX-MEM-GEN-KAN-STR-SXT	1	“Cheese CA-Dilution 10^-1 (2)”
	7	PEN-AMC-LEX-DOR-MEM-IPM-STR	1	“Cheese CA-Dilution 10^-1 (3)”
	9	PEN-AMP-AMC-CTX-LEX-DOR-MEM-IPM-SXT	1	“Cheese CA-Dilution 10^-3”
	8	PEN-AMP-AMC-FEP-LEX-IPM-GEN-STR	1	“Cheese EA-Dilution 10^-1 (1)”
	7	PEN-AMP-AMC-GEN-KAN-TET-CHL	1	“Cheese EA-Dilution 10^-1 (3) [RE]”
	7	PEN-AMP-AMC-CTX-LEX-IPM-STR	1	“Cheese GA-Dilution 10^-1 (3)”
	8	PEN-AMP-AMC-CTX-LEX-IPM-GEN-STR	1	“Cheese GA-Dilution 10^-1 (4)”
	10	PEN-AMP-AMC-LEX-DOR-MEM-STR-TET-CIP-SXT	1	“Cheese HA-Dilution 10^-1 (1) [RE]”
	9	PEN-AMP-AMC-DOR-MEM-GEN-STR-TET-SXT	1	“Cheese HA-Dilution 10^-1 (3) [RE]”

	12	PEN-AMP-AMC-FEP-CTX-LEX-CFM-DOR-MEM-GEN-NOR-SXT	1	“Cheese IA-Dilution 10^-1 (2)”
	14	PEN-AMP-AMC-FEP-CTX-LEX-CFM-DOR-MEM-GEN-KAN-STR-TET-SXT	1	“Cheese JA-Dilution 10^-3 (3)”
	8	PEN-AMP-AMC-LEX-GEN-KAN-TET-CHL	1	“Cheese JA-Dilution 10^-1 (1) [RE]”
	13	PEN-AMP-AMC-FEP-CTX-LEX-CFM-DOR-MEM-STR-TET-SXT-CHL	1	“Cheese LA-Dilution 10^-1 (1)”
	10	PEN-AMP-AMC-CTX-LEX-DOR-MEM-IPM-GEN-STR	1	“Cheese LA-Dilution 10^-1 (2)”
	8	PEN-AMP-AMC-KAN-STR-TET-SXT-CHL	1	“Cheese LA-Dilution 10^-1 (*)”
	5	PEN-AMP-AMC-GEN-SXT	1	“Cheese LA-Dilution 10^-1 (1) [RE]”
	7	PEN-AMC-CTX-LEX-IPM-GEN-SXT	1	“Cheese LA-Dilution 10^-1 (3) [RE]”
	10	PEN-AMP-AMC-CTX-LEX-DOR-MEM-IPM-KAN-STR	1	“Cheese MA(I)-Dilution 10^-1 (1)”
	11	PEN-AMP-AMC-FEP-CTX-LEX-DOR-IPM-GEN-KAN-STR	1	“Cheese MA(I)-Dilution 10^-1 (2)”
	9	PEN-AMP-AMC-GEN-KAN-STR-TET-SXT-CHL	1	“Cheese MA(I)-Dilution 10^-1 (4)”
	9	PEN-AMP-AMC-CTX-LEX-DOR-MEM-STR-SXT	1	“Cheese MAII-Dilution 10^-1 (1) [RE]”
	8	PEN-AMP-AMC-LEX-MEM-GEN-KAN-STR	1	“Cheese MAII-Dilution 10^-1 (2) [RE]”
	13	PEN-AMP-AMC-FEP-LEX-CFM-DOR-MEM-GEN-KAN-STR-TET-SXT	1	“Cheese NA-Dilution 10^-1 (2)”
	9	PEN-AMP-AMC-LEX-KAN-STR-TET-SXT-CHL	1	“Cheese NA-Dilution 10^-1 (*)”
	12	PEN-AMP-AMC-CTX-LEX-DOR-MEM-IPM-GEN-KAN-STR-CHL	1	“Cheese NA-Dilution 10^-1 (1) [RE]”
	9	PEN-AMP-AMC-DOR-MEM-GEN-STR-TET-CHL	1	“Cheese NA-Dilution 10^-1 (4) [RE]”

	5	PEN-AMP-AMC-LEX-GEN	1	“Cheese OA-Dilution 10^-1 (1)”
	8	PEN-AMP-AMC-LEX-STR-TET-SXT-CHL	1	“Cheese OA-Dilution 10^-1 (2)”
	6	PEN-AMC-LEX-TET-SXT-CHL	1	“Cheese OA-Dilution 10^-1 (1) [RE]”
	8	PEN-AMP-AMC-CTX-LEX-TET-SXT-CHL	1	“Cheese OA-Dilution 10^-1 (2) [RE]”
	3	PEN-AMC-LEX	1	“Cheese PA-Dilution 10^-3 (1)”
	11	PEN-AMC-FEP-LEX-CFM-DOR-MEM-KAN-STR-TET-CHL	1	“Cheese QA-Dilution 10^-1 (1)”
	5	PEN-AMP-AMC-CTX-LEX	1	“Cheese QA-Dilution 10^-3 (1)”
	5	PEN-AMP-AMC-GEN-STR	1	“Cheese QA-Dilution 10^-1 (1) [RE]”
	7	PEN-AMP-AMC-LEX-GEN-KAN-STR	1	“Cheese QA-Dilution 10^-1 (1) [RE]”
	3	PEN-AMC-STR	1	“Cheese RA-Dilution 10^-1 (2)”
	14	PEN-AMP-AMC-FEP-CTX-LEX-CFM-DOR-GEN-KAN-STR-TET-SXT-CHL	1	“Cheese SA-Dilution 10^-1 (1)”
	10	PEN-AMP-AMC-LEX-IPM-GEN-STR-TET-SXT-CHL	1	“Cheese UA-Dilution 10^-1 (1)”
	6	PEN-AMP-AMC-STR-TET-SXT	1	“Cheese UA-Dilution 10^-1 (2)”
	9	PEN-AMP-AMC-LEX-IPM-GEN-STR-TET-SXT	1	“Cheese UA-Dilution 10^-1 (3)”
	10	PEN-AMP-AMC-CTX-LEX-DOR-MEM-STR-TET-SXT	1	“Cheese VA-Dilution 10^-1 (1)”
	10	PEN-AMP-AMC-LEX-GEN-KAN-STR-TET-SXT-CHL	1	“Cheese VA-Dilution 10^-1 (3)”
	4	PEN-AMP-AMC-DOR	1	“Cheese WA-Dilution 10^-1 (1)”
	5	PEN-AMP-AMC-LEX-DOR	1	“Cheese WA-Dilution 10^-1 (3)”
Akkawi Cheese Brine	1	PEN	2	“Cheese B-Brine-Dilution 10^-1 (1)”, “Cheese B-Brine-Dilution 10^-1 (3)

	5	PEN-AMP-AMC-LEX-CFM	1	"Cheese B-Brine-Dilution 10^-1 (2)"
	14	PEN-AMP-AMC-FEP-CTX-LEX-CFM-DOR-MEM-IPM-STR-TET-NOR-SXT	1	"Cheese P-Brine-Dilution 10^-1 (2)"
	5	PEN-AMC-FEP-CTX-STR	1	"Cheese P-Brine-Dilution 10^-1 (1) [RE]"
	4	PEN-AMC-FEP-CTX	1	"Cheese P-Brine-Dilution 10^-1 (2) [RE]"
	13	PEN-AMP-AMC-CTX-LEX-CFM-MEM-GEN-KAN-STR-TET-SXT-CHL	1	"Cheese R-Brine-Dilution 10^-1 (3)"
	10	PEN-AMP-AMC-LEX-GEN-KAN-STR-TET-SXT-CHL	1	"Cheese R-Brine-Dilution 10^-1 (2)"
	10	PEN-AMP-AMC-FEP-GEN-KAN-STR-TET-SXT-CHL	1	"Cheese R-Brine-Dilution 10^-1 (1) [RE]"
	12	PEN-AMP-AMC-FEP-CTX-CFM-GEN-KAN-STR-TET-SXT-CHL	1	"Cheese R-Brine-Dilution 10^-1 (2) [RE]"
	10	PEN-AMP-AMC-CTX-GEN-KAN-STR-TET-SXT-CHL	1	"Cheese R-Brine-Dilution 10^-1 (3) [RE]"
	9	PEN-AMP-AMC-GEN-KAN-STR-TET-SXT-CHL	1	"Cheese R-Brine-Dilution 10^-1 (4) [RE]"
	8	PEN-AMP-AMC-CTX-LEX-DOR-MEM-IPM	1	"Cheese X-Brine-Dilution 10^-1 (2)"
	3	PEN-AMP-AMC	1	"Cheese AA-Brine-Dilution 10^-1 (1)"
	3	PEN-AMC-CTX	1	"Cheese AA-Brine-Dilution 10^-1 (2) [RE]"
	6	PEN-AMP-AMC-CTX-LEX-STR	1	"Cheese AA-Brine-Dilution 10^-1 (3) [RE]"
	8	PEN-AMP-AMC-CTX-LEX-STR-TET-SXT	1	"Cheese AA-Brine-Dilution 10^-1 (4) [RE]"

Table 4. Prevalence of Phenotypic Resistance of *Escherichia coli* strains isolated from the Akkawi cheese samples and from the brine samples

Of the 94 antibiotic resistance phenotypes of cheese isolates 12 phenotypes (12.8%) were recurring and shared amongst the strains. Of the 12 phenotypes the most frequent phenotype was PEN and it occurred in 8 *E. coli* isolates (6.8%) **(Table 4)**. Amongst the multidrug resistant (MDR) cheese isolates, the most frequent resistant phenotype was PEN-AMP-AMC-FEP-CTX-LEX-CFM-DOR-MEM-GEN-KAN-STR-TET-SXT-CHL and occurred in 4 cheese isolates (3.4%) **(Table 4)**. 82 isolates (69.5%) of the cheese isolates exhibited unique antibiotic resistance phenotypes. As for the brine isolates, of the 16 resistance phenotypes, only one (6.3%) which is PEN occurred in 2 brine isolates (11.8%) **(Table 4)**. The remaining 15 isolates (88.2%) exhibited unique resistance phenotypes **(Table 4)**. Of the resistance phenotypes of cheese isolates, 30 (31.9%) included at least 10 antibiotics. As for the brine isolates, of the resistance phenotypes 6 (37.5%) included at least 10 antibiotics **(Table 4)**.

Moreover, cheese isolate "Cheese VA-Dilution 10^-1 (3)" shared an identical antibiotic resistance phenotype with brine isolate "Cheese R-Brine-Dilution 10^-1 (2)", which is PEN-AMP-AMC-LEX-GEN-KAN-STR-TET-SXT-CHL. But also, cheese isolate "Cheese MA(I)-Dilution 10^-1 (4)" and brine isolate "Cheese R-Brine-Dilution 10^-1 (4) [RE]" shared an identical antibiotic resistance phenotype, which is PEN-AMP-AMC-GEN-KAN-STR-TET-SXT-CHL **(Table 4)**.

Furthermore, the overall multidrug resistant (MDR) *E. coli* isolates exhibited very high levels of resistance to critically important antibiotics, including amoxicillin-clavulanate (98.0%), ampicillin (81.4%), cephalexin (78.4%), streptomycin (71.6%), trimethoprim-sulfamethoxazole (59.8%), tetracycline (53.9%), gentamicin (51.0%), and cefotaxime (51.0%) **(Table 4)**.

C. *Staphylococcus aureus* PCR Confirmation and Methicillin-resistant Strains Identification

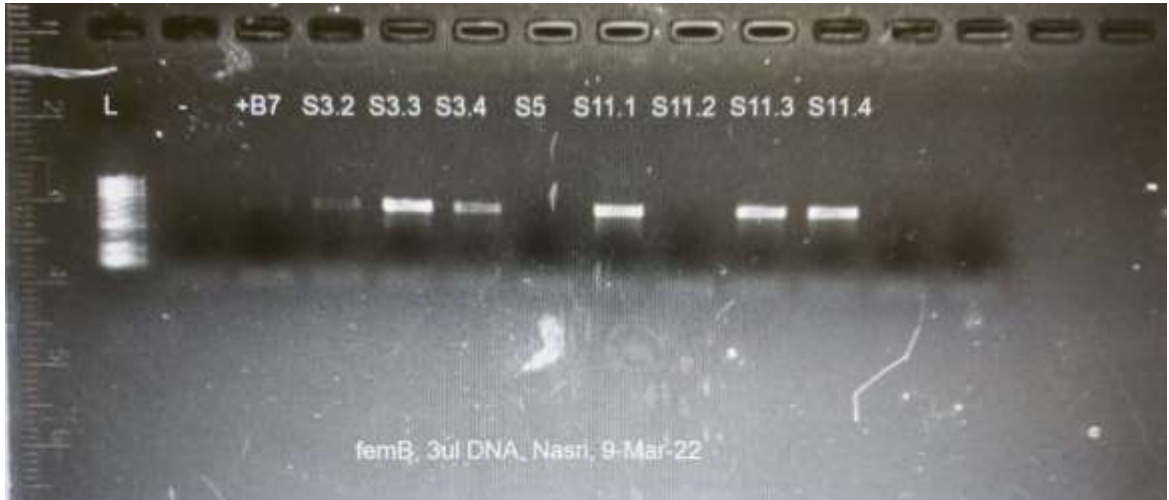


Figure 6. PCR signals for the *fem*B gene

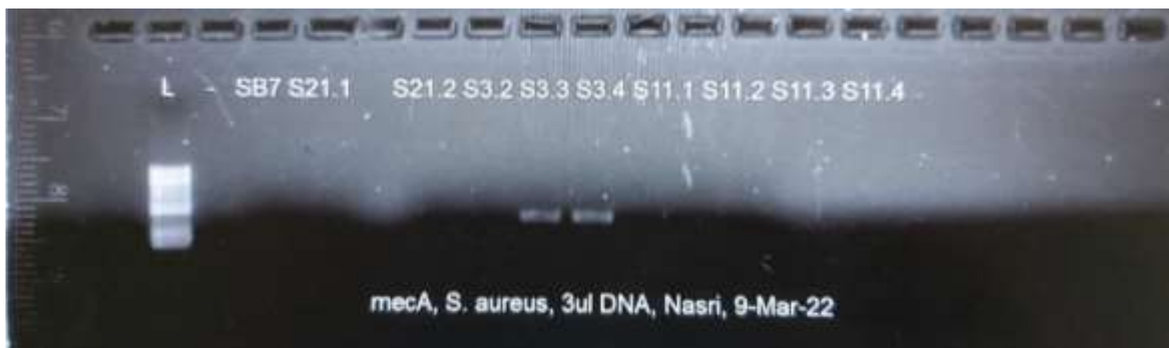


Figure 7. PCR signals for the *mec*A gene

Of the previously isolated *S. aureus* strains, 18 isolates were tested for the *femB* gene, due to their exhibiting the morphological characteristics of *S. aureus* colonies, which are a shiny black color and a halo around the colony. Upon testing for the *femB* a total of 9 isolates (50%) were confirmed to harbor the gene and were thus confirmed as *S. aureus* **(Figure 6)**. These 9 isolates were tested for the *mecA* gene to detect methicillin resistant strains amongst

them. Of the 9 isolates, 2 isolates (22.2%) harbored the *mecA* gene and were thus found to be methicillin-resistant strains **(Figure 7)**.

CHAPTER IV

DISCUSSION

Escherichia coli and fecal coliforms are used as indicators of fecal contamination in water and food. In fact, *E. coli* a bacterium that is part of the fecal coliforms is naturally present in the intestines of vertebrates. As such, the presence of *E. coli* in food or water is indicative that fecal contamination may have taken place and that other fecal microorganisms, including pathogens, may be present ("An Evaluation of the Role of Microbiological Criteria for Foods and Food Ingredients"). And thus, higher levels of *E. coli* and fecal coliforms imply a higher probability of the presence of pathogens. Pathogenic microorganisms that can potentially be present in white brine cheeses include bacterial foodborne pathogens such as, *Salmonella*, and *Listeria monocytogenes*. While *E. coli* is generally non-pathogenic some strains are pathogenic and can cause illness in humans. These pathogenic strains include the enterohemorrhagic *E. coli* also known as *E. coli* O157:H7. According to Osaili et al., *E. coli* O157:H7 was able to survive in cheese whether it is stored in 10% or 15% brine, at a temperature of 10ºC or 21ºC and regardless of the presence of starter culter or not (2014). The levels of *E. coli* and fecal coliforms in the tested Akkawi cheese samples were found to be extremely high reaching a maximum of 20,333,334 CFU/g and 75,500,000 CFU/g, respectively. The densities for *E. coli* were deemed very high and exceeded the maximum acceptable level. In fact, 86% and 100% of the Akkawi cheese were contaminated with levels of *E. coli* exceeding, respectively, the maximum acceptable levels of 1000 CFU/g and <10 CFU/g as per the LIBNOR standards for Akkawi cheese (2002:223) and (2003:495). As such the cheese samples were deemed microbiologically

unacceptable as per national standards since they are likely to harbor foodborne pathogenic bacteria.

Staphylococcus aureus is an indicator of hygiene. They are present in the nasal passages and on the skin of 25% of humans and animals (Centers of Disease Control and Prevention [CDC]). *S. aureus* in food are usually associated with extensive handling of the food ("An Evaluation of the Role of Microbiological Criteria for Foods and Food Ingredients"). This is the case of Akkawi cheese, since most of its production is done in traditional creameries and handling is done by hand. But also, *S. aureus* contamination can associated with environmental exposure or processing equipment (U.S. Food and Drug Administration [FDA]). In fact, as it was stated by Kümmel et al., some *S. aureus* from the farming setting are believed to be able to adapt to the dairy processing setting (2016). 87.5% of the Akkawi cheese were contaminated with levels of *S. aureus* exceeding, respectively, the maximum acceptable levels of 1000 CFU/g and 100 CFU/g as per the LIBNOR standards for Akkawi cheese (2002:223) and (2003:495). According to the U.S. Food and Drug Administration (FDA), staphylococcal enterotoxins (SE) have an infective dose of <1.0μg that is produced by 100,000 CFU/g. However, highly sensitive people can get ill at an infective dose as low 100-200ng (U.S. Food and Drug Administration [FDA]). One sample "Cheese SA" was contaminated with 1,583,334 CFU/g of *S. aureus*, exceeding the infective dose 100,000 CFU/g. As such the tested Akkawi cheese samples were deemed microbiologically unacceptable. But also, *S. aureus* is tolerant to a relativey high concentrations of salt (NaCl). And the presence and absence of starter culture as well as the storage temperature play a major role in the growth of *S. aureus* and its potential to produce enterotoxins. In a study, by Al-Nabulsi et al., it was found that a brine concentration of 15%

NaCl, the presence of starter culture, a storage temperature of 10ºC, and an acidic pH (~5.2) were found to reduce the growth of *S. aureus* and salt may have disable its enterotoxigenic potential (2020). This was in a way aligned with our results. In fact, we found a moderate positive linear association between the count of *S. aureus* and the pH of the brine solution. This shows that the decrease in pH that can be due to the production of lactic acid by lactic acid bacteria has an inhibitory effect on the growth of *S. aureus* (Al-Nabulsi et al., 2020). However, at a temperature of 25ºC higher growth rate of *S. aureus* was observed in cheese stored in 10% NaCl, in the absence of starter culture as well as in its presence (Al-Nabulsi et al., 2020). This shows that inadequate brining and storage conditions during the production of the white cheese such as Akkawi can result in an exponential growth of *S. aureus* and an enhancement of its enterotoxigenic activity. Furthermore, of the confirmed *S. aureus* isolates, 2 strains were found to be methicillin-resistant, which are isolates 3 and 4 from "Cheese C". "Cheese C" was originally vacuumed-packed, however, it didn't have a brand. This probably indicates that it was produced in a traditional dairy facility, and thus the MRSA strains originate from the handler's hands. But also, cross-contamination can be an alternative explanation since upon collecting "Cheese C" the retailer cut open the vacuum bag and cut a piece of the cheese mold. the knife he used cut can be contaminated with MRSA from another cheese sample or from the retailer's hand or environment.

On the other hand, *Escherichia coli* is also used as an indicator of antibiotic resistance. Our findings have shown a high prevalence of 77.1% of multidrug resistant (MDR) *E. coli* amongst strains isolated from cheese samples. The resistance phenotypes were quite diverse, accounting for 84 unique antibiotic resistance phenotypes only amongst the MDR cheese strains. Some *E. coli* strains even showed a wide phenotypic resistance profile.

In fact, 4 *E. coli* strains exhibited resistance to 15 different antibiotics. According to Anjum et al., antimicrobial resistance (AMR) diversity is more common in *E. coli* wastewater isolates (2021). This suggests that a high proportion of *E. coli* strains isolated from the cheese samples share similar phenotypic resistance patterns with wastewater *E. coli* isolates. What further suggests wastewater contamination is the relatively high prevalence of resistance against β-lactams, including penicillins with high rates of resistance against amoxicillin-clavulanate and ampicillin, respectively 88.1% and 67.8%, and the 3rd generation cephalosporin cefotaxime with resistance rate of 40.7%. In fact, according to Delgado-Blas et al., high-level resistance to β-lactams was common amongst wastewater isolates and is associated with the presence of the extended β-lactamase (ESBL) genes *bla*$_{CTX\text{-}M\text{-}55}$ and *bla*$_{CMY\text{-}2}$ genes (2021). Furthermore, resistance against aminoglycosides was also found to be characteristic to wastewater isolates and is indicative of anthropogenic pressure and heavy use of aminoglycosides in clinical treatments (Delgado-Blas et al., 2021). Resistance to aminoglycosides was observed amongst the *E. coli* strains isolated from Akkawi cheese samples. Resistance to aminoglycosides was relatively high with highest being against streptomycin (57.6%), followed by gentamicin (40.7%), and lastly kanamycin (35.6%). Furthermore, the high-level resistance against streptomycin can also be explained by the extensive use of this antibiotic in farming practices (Kassaify et al., 2013). In addition, according to Dandachi et al., gentamicin resistant *E. coli* were significantly associated with the use of Gentamicin as food additive in poultry feed (2018). But also, relatively high resistance to tetracycline amongst *E. coli* cheese isolates, with a rate of 39.8%. This can also be due to the abuse of tetracycline in the farming sector. According to Jammoul and El Darra, the maximum level of tetracycline residues detected in tested broiler chicken carcasses was

63.8µg/kg with a mean level of 24.4µg/kg (2019). This can be indicative that most *E. coli* isolates from Akkawi cheese originate from farm, hospital, and/or domestic effluents. But also, in a study by Vranic and Uzunovic a resistance rate against trimethoprim-sulfamethoxazole was found to be 40.86% and was deemed the second highest (2016). This resistance rate was close to the resistance rate against trimethoprim-sulfamethoxazole in *E. coli* strains isolated from Akkawi cheese of 46.6%. According to Vranic and Uzunovic, trimethoprim-sulfamethoxazole is heavily used to treat urinary tract infections (2016). It was further stated that the resistance rate against Trimethoprim-Sulfamethoxazole in developing countries usually ranges between 30 and 40% (Vranic and Uzunovic, 2016). And it also aligns with the status of Lebanon as a developing country. Furthermore, according to Diab et al., in Lebanon, untreated domestic, farm, and hospital effluents are discarded into natural water receptacles without prior treatment (2018). Indeed, wastewater contamination of the cheese could have occurred directly through the use of contaminated water to make the brine solution. This is evident by the sharing of an identical phenotypic resistance profile between isolate "Cheese R-Brine-Dilution 10^-1 (2)" and isolate "Cheese VA-Dilution 10^-1 (3)" and between isolate "Cheese R-Brine-Dilution 10^-1 (4) [RE]" and isolate "Cheese MA(I)-Dilution 10^-1 (4)". The fact different that non-complementary brine and cheese *E. coli* isolates share an identical phenotypic resistance profile can suggest a common origin. But also, indirect contamination may have occurred by washing the equipment with contaminated water or the cheese handler washing his hand with contaminated water.

CHAPTER V

CONCLUSION

Akkawi cheese is a white brine cheese that is popular in the Middle East and especially in Lebanon. While it is used as an ingredient in some cooked dishes, Akkawi cheese is consumed raw like many other white brined cheeses. And due to the predominance of small traditional dairy facilities in Lebanon, food safety concerns arise when it comes to the preparation and handling of Akkawi cheese. Our findings have revealed that Akkawi cheeses, mainly those produced by hand, are contaminated with relatively high levels of *Escherichia coli* and fecal coliforms, which are indicators of fecal pollution. But also, high levels of hygiene indicator and foodborne pathogen *Staphylococcus aureus* were detected. The level of contamination of both *E. coli* and *S. aureus* exceeded the maximum acceptable level as per the national standard LIBNOR. As such, the tested cheese samples are deemed microbiologically unacceptable. And thus, it is advisable not consume this type of cheese raw. Thermal processing of moderately contaminated Akkawi cheese can reduce microbiological contaminants to an acceptable level. But also, the tested Akkawi samples revealed serious nationwide food safety-related problems including antibiotic resistance and environmental pollution. In fact, a high proportion (77.1%) of *E. coli* strains isolated from Akkawi cheese were found to be multidrug resistant (MDR). Furthermore, these MDR strains seemed to exhibit phenotypic antibiotic resistance patterns that are characteristic to wastewater isolates. These phenotypic patterns included relatively high resistance to β-lactams, aminoglycosides, and trimethoprim-sulfamethoxazole, and tetracycline. These resistance phenotypes indicated the potential direct or indirect contamination of the cheese

production with wastewater due to heavy environmental pollution. But also, relatively high streptomycin and gentamicin resistance amongst *E. coli* was indicative of poor antibiotic stewardship and abuse in the farming sector. Whilst the detection of MRSA in the Akkawi cheese sample is indicative of the dissemination and circulation of antibiotic resistance within the community and the ecosystem. As such, this problem should be dealt with using a One Health approach, in that there should be a collaboration between different ministries, including the Lebanese Ministry of Public Health, the Lebanese Ministry of Agriculture, and the Lebanese Ministry of Economy and Trade. But also, a collaboration of the public and private sectors is needed to enhance the testing of different food samples and develop strategies to limit sewage environmental pollution, such as establishing and enhancing wastewater treatment plants. In addition, policies that regulate the use of antibiotics in different sectors and restricts the use of critically important antibiotics to the public health sector. Failure to do so will likely result in a major public health and economic crisis.

REFERENCES

Alhomoud, F., Aljamea, Z., Almahasnah, R., Alkhalifah, K., Basalelah, L., & Alhomoud, F.K. (2017). Self-medication and self-prescription with antibiotics in the Middle East—do they really happen? A systematic review of the prevalence, possible reasons, and outcomes. *International Journal of Infectious Diseases, 57*,3-12. Retrieved from *ScienceDirect*. https://doi-org.ezproxy.aub.edu.lb/10.1016/j.ijid.2017.01.014

Al-Nabulsi, A.A., Osaili, T.M., Abu Naser, R.A., Olaimat, A.N., Ayyash, M., Al-Holy, M.A., Kadora, K.M., & Holley, R.A. (2020). Factors affecting the viability of Staphylococcus aureus and production of enterotoxin during processing and storage of white-brined cheese". *Journal of Dairy Science,* 103:6869-6881. https://doi.org/10.3168/jds.2020-18158

Anjum, M.F., Schmitt, H., Börjesson, S., Berendonk, T.U. (2021). The potential of using *E. coli* as an indicator for the surveillance of antimicrobial resistance (AMR) in the environment. *Current Opinion in Microbiology, 64*, 152-158. Retrieved from *ScienceDirect.* https://doi-org.ezproxy.aub.edu.lb/10.1016/j.mib.2021.09.011

Bou-Mitri, C., Mahmoud, D., El Gerges, N., & Abou Jaoude, M. (2018). Food safety knowledge, attitudes, and practices of food handlers in Lebanese hospitals: A cross-sectional study. *Food Control, 94*(2018), 78-84. Retrieved from *ScienceDirect.*
https://doi.org/10.1016/j.foodcont.2018.06.032

Castro, A., Santos, C., Meireles, H., Silva, J., & Teixeira, P. (2016). “Food handlers as potential sources of dissemination of virulent strains of *Staphylococcus aureus* in the community”. *Journal of Infection and Public Health, 9*(2), 153-160. Retrieved from *ScienceDirect.*
https://doi.org/10.1016/j.jiph.2015.08.001

Centers of Disease Control and Prevention. *Carbapenem-resistant Enterobacterales (CRE).* Retrieved from https://www.cdc.gov/

Centers of Disease Control and Prevention. *ESBL-producing Enterobacteriaceae in Healthcare Settings.* Retrieved from https://www.cdc.gov/

Centers of Disease Control and Prevention. *Klebsiella pneumoniae in Healthcare Settings*. Retrieved from https://www.cdc.gov/

Cosandey, A., Boss, R., Luini, M., Artursson, K., Bardiau, M., Breitenwieser, F., Hehenberger, E., Lam, Th., Mansfeld, M., Michel, A., Mösslacher, G., Naskova, J., Nelson, S., Podpecan, O., Raemy, A., Ryan, E., Salat, O., Zangerl, P., Steiner, A., & Graber, H.U. (2016). *Staphylococcus aureus* genotype B and other genotypes isolated from cow milk in European countries. *Journal of Dairy Science, 99*(1), 529-40. Retrieved from *ScienceDirect.*
https://doi.org/10.3168/jds.2015-9587

Dandachi, I., Sokhn, E.S., Dahdouh, E.A., Azar, E., El-Bazzal, B., Rolain, J.-M., & Daoud, Z. (2018). Prevalence and Characterization of Multi-Drug-Resistant Gram-Negative Bacilli Isolated From Lebanese Poultry: A Nationwide Study. *Frontiers in Microbiology, 9*:550. Retrieved from *PubMed Central.*
https://doi.org/10.3389/fmicb.2018.00550

Dankar, I., Hassan, H., & Serhan, M. (2022). Knowledge, attitudes, and perceptions of dairy farmers regarding antibiotic use: Lessons from a developing country. *Journal of Dairy Science, 105*(2), 1519-1532. Retrieved from *ScienceDirect.* https://doi.org/10.3168/jds.2021-20951

Delgado-Blas, J.F., Ovejero, C.M., David, S., Montero, N., Calero-Caceres, W., Garcillan-Barcia, M.P., de la Cruz, F., Muniesa, M., Aanensen, D.M., & Gonzalez-Zorn, B. (2021). Population genomics and antimicrobial resistance dynamics of *Escherichia coli* in wastewater and river environments. *Communications Biology, 4* :457(2021). Retrieved from *Nature.*

Diab, M., Hamze, M., Bonnet, R., Saras, E., Madec J.-Y., & Haenni, M. (2018). Extended-spectrum beta-lactamase (ESBL)- and carbapenemase-producing Enterobacteriaceae in water sources in Lebanon. *Veterinary Microbiology, 217*(2018), 97-103. Retrieved from *ScienceDirect.* https://doi-org.ezproxy.aub.edu.lb/10.1016/j.vetmic.2018.03.007

Diab, M., Hamze, M., Bonnet, R., Saras, E., Madec, J.-Y., & Haenni, M. (2017). OXA-48 and CTX-M-15 extended-spectrum beta-lactamases in raw milk in Lebanon: epidemic spread of dominant *Klebsiella pneumoniae* clones. *Journal of Medical Microbiology, 66*(11):1688-1691. Retrieved from *ScienceDirect.* https://doi.org/10.1099/jmm.0.000620

Jammoul, A., & El-Darra, N. (2019). Evaluation of Antibiotics Residues in Chicken Meat Samples in Lebanon. *Antibiotics, 8*(2):69. Retrieved from *MDPI.* https://dx.doi.org/10.3390%2Fantibiotics8020069

Kassaify, Z., Abi Khalil, P., & Sleiman, F. (2013). Quantification of Antibiotic Residues and Determination of Antimicrobial Resistance Profiles of Microorganisms Isolated from Bovine Milk in Lebanon. *Food and Nutrition Sciences, 4*, 1-9. http://dx.doi.org/10.4236/fns.2013.47A001

Kassem, I.I., Hijazi, M.A., & Saab, R. (2019). On a collision course: The availability and use of colistin-containing drugs in human therapeutics and food-animal farming in Lebanon. *Journal of Global Antibiotic Resistance,16*, 162-164. Retrieved from *ScienceDirect.* https://doi-org.ezproxy.aub.edu.lb/10.1016/j.jgar.2019.01.019

Kharroubi, S., Nasser, N.A., El-Harakeh, M.D., Alhaj Sulaiman, A., & Kassem, I.I. (2020). First Nation-Wide Analysis of Food Safety and Acceptability Data in Lebanon. *Foods*, *9*(11), 1717. Retrieved from *MDPI.* https://doi.org/10.3390/foods9111717

Kümmel, J., Stessl, B., Gonano, M., Walcher, G., Bereuter, O., Fricker, M., Grunert, T., Wagner, M., & Ehling-Schulz, M. (2016). Staphylococcus aureus Entrance into the Dairy Chain: Tracking S. aureus from Dairy Cow to Cheese. *Frontiers in Microbiology.* https://doi.org/10.3389/fmicb.2016.01603

Lebanese Ministry of Public Health. Food Safety. https://www.moph.gov.lb/en/Pages/4/126/food-safety

National Research Council (US) Subcommittee on Microbiological Criteria (1985). *An Evaluation of the Role of Microbiological Criteria for Foods and Food Ingredients.* National Academy Press (US).

Nicolas-Chanoine, M.-H., Bertrand, X., & Madec, J.-Y. (2014). Escherichia coli ST131, an Intriguing Clonal Group. *Clinical Microbiology Reviews*, *27*(3), 543-574. Retrieved from *PubMed Central.* https://dx.doi.org/10.1128%2FCMR.00125-13

Osaili, T.M., Al-Nabulsi, A.A., Olaimat, A.N., Shaker, R.R., Taha, M., & Holley, R.A. (2014). Survival of Escherichia coli O157:H7 during manufacture and storage of white brined cheese. *Journal of Food Science, 79*(9):M1750-5. Retrieved from *PubMed National Library of Medicine (NIH).* https://doi.org/10.1111/1750-3841.12547

Osman, M., Kamal-Dine, K., El Omari, K., Rafei, R., Dabboussi, F., & Hamze, M. (2019). Prevalence of *Staphylococcus aureus* methicillin-sensitive and methicillin-resistant nasal carriage in food handlers in Lebanon: a potential source of transmission of virulent strains in the community. *Access Microbiology, 1*(6): e000043. Retrieved from *PubMed Central.* https://dx.doi.org/10.1099%2Facmi.0.000043

Saadeh, M., & Wakim, E. (2017). Deterioration of Groundwater in Beirut Due to Seawater Intrusion. *Journal of Geoscience and Environment Protection, 5*(11):149-159. Retrieved from *ResearchGate.* http://dx.doi.org/10.4236/gep.2017.511011

Sergelidis, D., & Angelidis, A.S. (2017). Methicillin-resistant Staphylococcus aureus: a controversial food-borne pathogen. *Letters in Applied Microbiology, 64*(6): 409-418. https://doi.org/10.1111/lam.12735

Schnitt, A., & Tenhagen, B.-A. (2020). Risk Factors for the Occurrence of Methicillin-Resistant *Staphylococcus aureus* in Dairy Herds: An Update. *Foodborne Pathogens and Disease, 17*(10): 585–596. https://dx.doi.org/10.1089%2Ffpd.2019.2638

U.S. Food and Drug Administration. *Bad Bug Book 2nd edition.*

Vranic, S.M., & Uzunovic, A. (2016). ANTIMICROBIAL RESISTANCE OF *ESCHERICHIA COLI* STRAINS ISOLATED FROM URINE AT OUTPATIENT POPULATION: A SINGLE LABORATORY EXPERIENCE. *Materia Socio Medica, 28*(2):121-124. Retrieved from *PubMed Central.* https://doi.org/10.5455/msm.2016.28.121-124

World Health Organization (2015). From kebabs to Fattoush – keeping Lebanon's food safe. https://www.who.int/features/2015/lebanon-food-safety/en/

World Health Organization (2016). WHO Critically Important Antimicrobials for Human Medicine 5th revision. https://www.who.int/foodsafety/publications/cia2017.pdf