LEBANESE AMERICAN UNIVERSITY

Processing Effects on the Oxytetracycline and Tylosin Antibiotic Residues in Middle Eastern Dairy Products

By

Liz Saidy

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Student Name: Liz Saidy I.D. #: 201402138

Thesis Title: Processing Effects on the Tetracycline and Tylosin Antibiotic Residues in Middle Eastern

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The undersigned certify that they have examined the final electronic copy of this thesis and approved it in Partial Fulfillment of the requirements for the degree of:

MSc in the major of Nutrition

Thesis Advisor's Name: Hussein Hassan

Signature: [redacted] Date: 26 / 08 / 20
Day Month Year

Committee Member's Name: Chadi Hosri

Signature: [redacted] Date: 26 / 08 / 20
Day Month Year

Committee Member's Name: Mireille Serhan

Signature: [redacted] Date: 26 / 08 / 20
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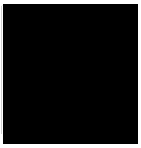
Signature: 

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Processing Effects on the Tetracycline and Tylosin Antibiotic Residues in Middle Eastern Dairy Products

Liz Saidy

ABSTRACT

Antibiotics are used extensively in healthcare and veterinary fields to treat or prevent certain diseases caused by infectious agents. Their presence in food supply poses a human health threat and thus, can negatively affect the food industry. The main objective of our first-of-its-kind study in the Middle East was to assess the effect of different processes (skimming, pasteurization, curding, pressing, salting, cheese boiling, and whey acidification and heating) on two widely used antibiotics in Lebanon, Oxytetracycline (OTC) and Tylosin (TYL), in making commonly consumed Middle Eastern cheeses (*Baladi, Double Crème, Halloum and Akkawi*). This was done by spiking 450 Kgs of full-fat bovine milk with 25 ppm/kg of OTC and 8 ppm/kg of TYL, then skimming and pasteurizing using the two common pasteurization methods (Holder and High Temperature Short Time (HTST)), and processing the milk to different cheeses. Liquid Chromatography Mass Spectrometry (LCMS), the gold standard for the quantification of antibiotics, was used. OTC and TYL were not homogenously distributed between the dairy products. Skimming decreased significantly ($p=0.015$) TYL concentration by 68.6%. OTC degradation during Holder proved to be significant ($p=0.015$) and ranged from 40 to 48%, while that during HTST processing (18%) was not significant. As for TYL, HTST had a significant ($p=0.012$) effect with 32% degradation, while holder pasteurization did not cause a significant degradation (26%). Curding step had a significant ($p=0.028$) effect on OTC only with the concentration increasing by almost 1.5 folds. Acidification and heating of whey to produce Double Cream decreased significantly ($p=0.037$) OTC concentration by 14.7 to 46.3%, while TYL concentration increased significantly ($p=0.000$) by 300%. Pressing and salting did not have any significant effect on OTC and TYL, while cheese boiling in making Halloum significantly decreased the concentration of both antibiotics. The highest concentrations of OTC were measured in curd-based cheeses that did not undergo boiling (Baladi and Akkawi), making them of higher risk in this regard. As for TYL, the highest transfer level was for the whey-based Double Cream, making this cheese of higher concern for this antibiotic.

Key words: Middle Eastern cheeses, OTC, TYL, Antibiotics, LCMS.

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LIST OF ABBREVIATIONS

CAN: Acetonitrile

DC: Doxycycline

CDC: Centers for Disease Control

EU: European Union

FDA: Food and Drug Administration

HTST: High Temperature Short Term

HPLC: High Performance Liquid Chromatography

OTC: Oxytetracycline

LCMS: Liquid Chromatography Mass Spectrometry

LLE: Liquide-Liquide Extraction

MMISPE: Matrix Solid-phase Dispersion Solid Phase Extraction

MRL: Maximum Residue Limit

LOQ: Limit of Quantification

PLE: Pressur\ized Liquid Extraction

PVDF: Polyvinylidene Fluoride

SPE: Solid Phase Extraction

SPME: Solid-Phase Microextraction

TCA: Trichloroacetic Acid

TYL: Tylosin

Chapter 1

Literature Review

1.1 Background

Antibiotics are used in both humans and animals for the treatment and prevention of several diseases led by infectious agents. Healthcare and veterinary fields have been relying on them since the discovery of the first antibiotic in the 1940s (Groot *et al.*, 2016). Over the past decade, antibiotic consumption has increased dramatically (Klein *et al.*, 2018), and with that, antibiotic resistance emerged, resulting in an increasing global crisis in this regard (Krömker *et al.*, 2017). Greater consumption of antibiotics was seen more evident in developing countries rather than developed ones (Krömker *et al.*, 2017). In Lebanon, the use of antibiotics has been emerging for medical use in humans as well as in the veterinary field. Worldwide, about 50% of all antibiotics made are used in animal agriculture applications (Kabrite *et al.*, 2019).

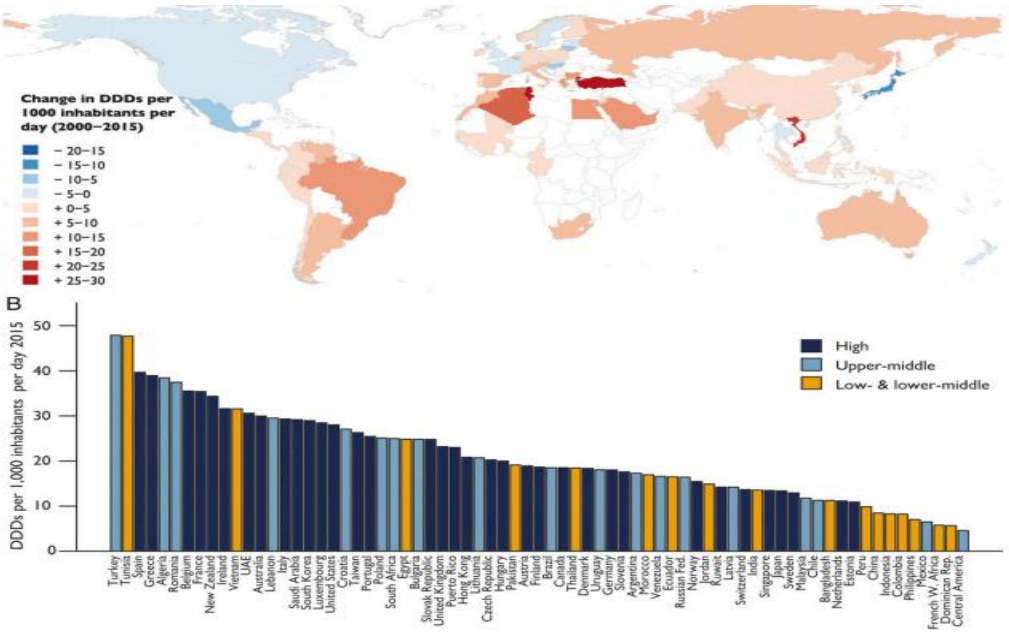


Fig. 1. Global antibiotic consumption by country: 2000–2015. (*A*) Change in the national antibiotic consumption rate between 2000 and 2015 in DDDs per 1,000 inhabitants per day. For Vietnam, Bangladesh, The Netherlands, and Croatia, change was calculated from 2005, and for Algeria from 2002 as data before those years for those countries were not available. (*B*) Antibiotic consumption rate by country for 2015 in DDDs per 1,000 inhabitants per day. Data source: IQVIA MIDAS, 2000–2015, IQVIA Inc. All rights reserved (https://www.iqvia.com/solutions/commercialization/geographies/midas).

(Adapted from Klein et al., *Proceedings of the National Academy of Sciences*, 2018)

Figure 1. 2000-2015: Global antibiotic consumption by country

1.2 Animal Milk and Milk Products

The present and foreseen development of the total population calls for expanding high-quality animal protein production. Dairy farming is seen, particularly in developing countries, as one of the most important ways of fulfilling this need (Groot *et al.*, 2016). Milk offers a high-quality nutrition to all age groups owing to its elevated content of micro- and macronutrients, such as calcium, selenium, magnesium, zinc, riboflavin, pantothenic acid, and vitamin B12 (FAO, 2013).

The flavor, color and composition of the milk products is influenced by the class of the dairy animal, age, and intake, together with the lactation phase, farming system, parity,

season and physical environment. In the case of cow's milk (bovine milk), solid content has around 3-4% fat, 3.5% protein and 5% lactose, all of which is influenced by the variations mentioned previously (FAO, 2013).

Moreover, dairy products display large nutritional, compositional and cultural inconsistencies (Raza & Kim, 2018). This including butter, cheese, frozen desserts, ice cream, and milk powders. The Middle East has its unique white cheese selection such as Akkawi, Double Crème, Halloum, among others, which are deemed part of a healthy diet as an abundant source of protein, calcium, and other essential nutrients (Raza & Kim, 2018).

In the Middle East, there is an ascending trend of the proportion of energy intake from milk and dairy products. However, milk consumption did not reach the recommended daily intake (2-3 servings/day), averaging at 1.1 servings/day (Golzarand *et al*., 2012). Furthermore, in a cross-sectional study looking at dietary patterns in Lebanon, milk and dairy products provided 243.1 g/day (10% of total daily energy intake). Yogurt was the most consumed, followed by cheese, then labneh, while milk was the least consumed (with 43.2% of subjects reported not consuming milk) (Nasreddine *et al.*, 2006).

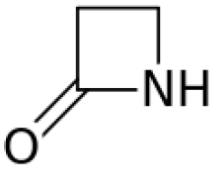
The modern consumer has gotten more mindful of the composition of food and the risks related with elevated intakes of specific food components. The routine testing of milk is needed to succeed in cheese making. In order to avoid the contamination of dairy products, several systems of milk control have to be employed. Thus, the dairy industry must ensure that milk and milk products are suitable, marketable, nutritious and free from chemical hazards, including antibiotic residues (Gajda *et al*. 2017).

1.3. Antibiotics:

Antibiotics, also known as antibacterial, are a group of drugs applied to cure a variety of infections caused by bacteria (Moir *et al*. 2012). The first discovered antibiotic was Penicillin by English Bacteriologist "Sir Alexander Fleming", and it was unintentionally obtained from fungus that inhibits the soil inhabiting "*Penicillium notatum".* The term antibiotics originates from "antibiosis" meaning "against life" (Etebu & Arikekpar, 2016). Several medical breakthroughs rely on the ability to use antibiotics to fight infections, such as cancer therapy, organ transplants, pneumonia, and sepsis (Moir et al. 2012). On the other hand, antibiotics do not treat infections that are viral such as the flu. They are commonly classified based on their mode of action, molecular structures and spectrum of activity (Walsh & Wencewicz, 2016). Antibiotics can be divided into five categories through their mode of action: nucleic acid synthesis inhibitors, protein synthesis inhibitors, cell membrane integrity disrupters, cell wall synthesis inhibitors, and metabolic pathway inhibitors (Walsh & Wencewicz, 2016). Another way of classification is based on the spectrum of activity. For instance, broad spectrum antibiotics, such as macrolides and tetracyclines, act against a broad range of microorganisms, and thus used where the specific type of the microorganism is still unknown (Walsh & Wencewicz, 2016). On the other hand, narrow spectrum antibiotics act against selective types of microorganisms being more effective on specific microorganisms but less effective on others. These are used only when the specific bacterial microorganism has been isolated and found to be sensitive to a specific antibiotic such as penicillin G and vancomycin (Moir *et al*. 2012). Furthermore, antibiotics may be categorized as bactericidal meaning they inhibit the cell wall synthesis of bacteria and cause their destruction. Examples on bactericidal antibiotics

include daptomycin, fluoroquinolones, nitrofurantoin, and metronidazole. In contrast, bacteriostatic antibiotics prohibit the bacteria's development, either by intervening with the production of bacterial protein, replication of DNA, or other parts of bacterial cellular metabolism. Examples include sulfonamides, tetracyclines, macrolides, chloramphenicol, and glycosamides (Walsh & Wencewicz, 2016). Selection of antibiotics depends largely on clinical manifestation of the infection as well as the patients' profile. The Kirby-Bauer method is one of the most commonly performed tests that helps to guide the selection of an effective antibiotic (Walsh & Wencewicz, 2016). In relation to their structural classification, antibiotics in the same class mostly demonstrate comparable forms of efficacy and negative side effects (Etebu & Arikekpar, 2016).

*Beta-lactams***.** This class of antimicrobials contains a 3-carbon and 1-nitrogen ring that is greatly reactive (Fig. 2). Beta-lactams meddle with the cell wall synthesis of bacteria. They include Penicillin, and the most commonly known and used Penicillins are Monobactams and Cephalosporins (Etebu & Arikekpar, 2016).



(Adapted from Etebu & Arikekpar, *Int. J. Appl. Microbiol. Biotechnol*, 2016)

Figure 2. Beta-lactam ring chemical structure

Penicillins. Penicillins are implicated in a class of different group of compounds, almost all of them end with "cillin". They are beta-lactam compounds comprising a nucleus of 6-animopenicillanic acid -lactam plus thiazolidine- ring and ring side chains. Members of this class include Penicillin G, Amoxicillin, Nafcillin, Methicillin and Ticarcillin (Boundless, 2016).

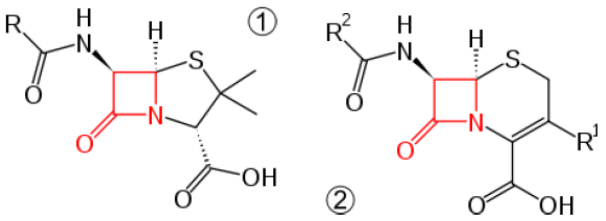


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Figure 3. Chemical structure of beta-lactam. Core structure of penicillins (right) and cephalosporins

Cephalosporin. Members of this class are part of the most frequently prescribed and dispensed antibiotics. This class of antibiotics contains 7-aminocephalosporanic acid nucleus and side chain containing 3,6-dihydro-2 H-1,3-thiazane rings (Fig. 4). Cephalosporins are applied to cure bacterial infections and diseases that rise from bacteria that produce Penicillinase, including some Escherichia coli, Proteus mirabilis, Methicillin-susceptible Staphylococci and Streptococci, Haemophilus influenza, Klebsiella pneumonia, Enterobacter aerogenes and some Neisseria (Etebu & Arikekpar, 2016).

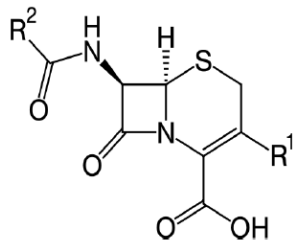

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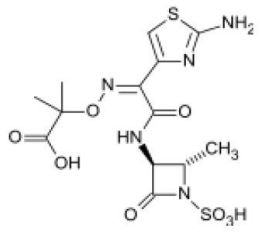
Figure 4. Structure of Cephalosporins

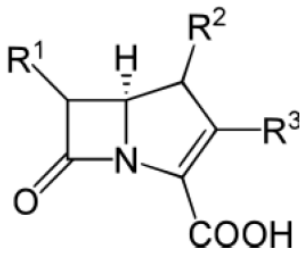
Monobactams. This class of antibiotics was acquired from the bacterium *Chromobacterium violaceum*. They are considered part of beta-lactam compounds; however not like most other beta-lactams, the beta-lactam ring of monobactams holds alone and is not attached to another ring (Fig. 5). Aztreonam is the only available commercial form of monobactam, which has a narrow spectrum of activity, applied for curing septicemia, urinary tract infections, and pneumonia. (Fu *et al.*, 2016).



(Adapted from Etebu & Arikekpar, *Int. J. Appl. Microbiol. Biotechnol*, 2016)

Figure 5. Structure of Monobactam

Carbapenems. Carbapenems, illustrated in Fig. 6, has a major part in fighting infections arising from bacteria. They are given when patients with infections come to be critically sick or are believed to harbor bacteria that is resistant and are often called "antibiotics of last resort (Livermore *et al.*, 2011; Patel and Bonomo, 2011).

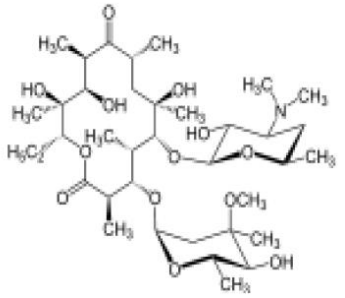


(Adapted from Walsh & Wencewicz, *American Society for Microbiology*, 2016)

Figure 6. Structure of Carbapenem

Macrolides. This class of antibiotics is described by deoxy sugars L-cladinose and D-desosamine attachments by the 14-, 15-, or 16- membered macrocyclic lactose rings (Fig. 7).

Members of this class work by inhibiting the synthesis of bacterial proteins. However, Macrolides are recycled into bile by the liver and thus have a tendency to accumulate in the body. In addition, they may produce inflammation. Hence, clinicians generally advise dispensing low doses of this drug. Example of macrolides include Erythromycin, Clarithromycin and Azithromycin (Tian *et al*. 2017).

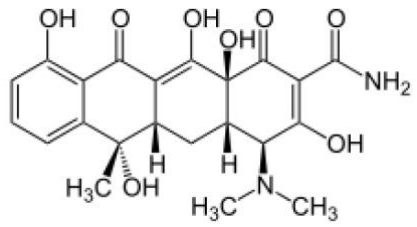


(Adapted from Walsh & Wencewicz, *American Society for Microbiology*, 2016)

Figure 7. Structure of Macrolide

Tetracyclines. Tetracyclines (TCs) contain four hydrocarbon rings (Fig. 8) (Fuoco, 2012). Members of this class are broad-spectrum drugs applied in human and veterinary treatments. They act by targeting the ribosome of the bacteria. Tetracyclines are characterized by their exceptional chemotherapeutic efficiency to treat a wide range of Gram-negative and Gram-positive bacteria, rickettsiae, large viruses, spirochetes, chlamydia, and protozoan parasites (Samanidou *et al.*, 2007). When it comes to side effects, the use of Tetracyclines has been associated with gastro-intestinal disturbance, photosensitivity, and hepatotoxicity. Furthermore, they have a strong affinity to Calcium

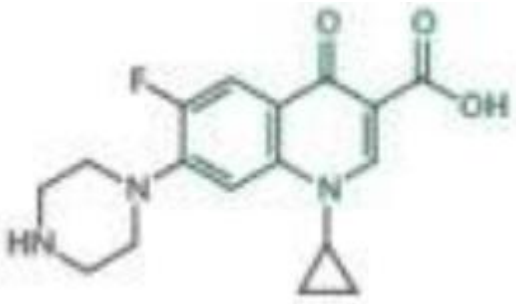
and can accumulate in developing teeth and bones leading to discoloration of teeth and inhibition of bone growth (Cabizza *et al*. 2017).



(Adapted from Walsh & Wencewicz, *American Society for Microbiology*, 2016)

Figure 8. Structure of Tetracycline

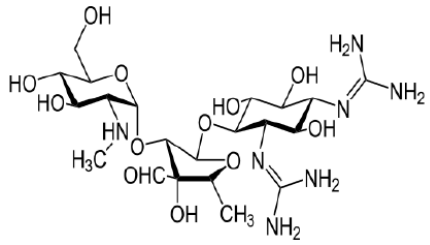
Quinolones. This class can affect the replication of DNA and the bacteria's transcription. Quinolones mostly consists of two rings (fig. 9). An additional ring was added to recent generations, which allows to have a broader range of antimicrobial activity, anaerobic bacteria (these were resistant to quinolones in the past). In vitro studies have seen a lot of progress; however, there is still a lack in understanding of the dynamics of toxicity between some of the antibiotics of this class (Walsh & Wencewicz, 2016).



(Adapted from Walsh & Wencewicz, *American Society for Microbiology*, 2016)

Figure 9. Structure of Quinolones

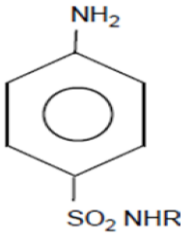
Aminoglycosides. Members of this class are compounds of usually 3-amino sugars linked by glycosidic bonds (Fig. 10). Aminoglycosides can hamper the synthesis of proteins in bacteria and are active versus aerobic Gram-negative strains and several Gram-positive bacteria. They are characterized by a broad spectrum of antimicrobial activity (Walsh & Wencewicz, 2016).



(Adapted from Walsh & Wencewicz, *American Society for Microbiology*, 2016)

Figure 10. Structure of Aminoglycoside (Streptomycin)

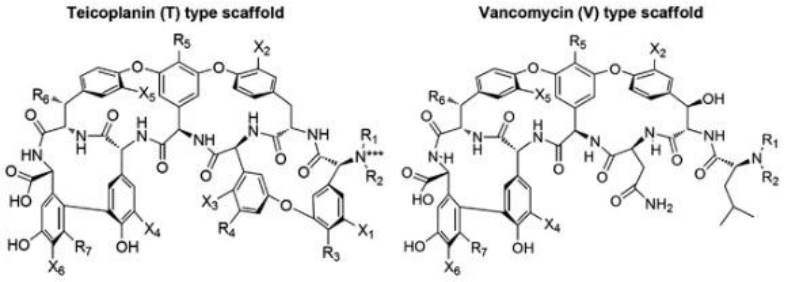
Sulphonamides. Sulphonamides are artificial antibacterial agents that contain the Sulphonamide group (Fig. 11) This class of antibiotics is the first one applied in medicine. They still hold an important role in medicine and veterinary practice. This class of antibiotics inhibits both Gram-negative and Gram-positive bacteria. They are commonly applied broadly in the treatment of several infections (Stawinski *et al*., 2013; Xu *et al*., 2014).



(Adapted from Walsh & Wencewicz, *American Society for Microbiology*, 2016)

Figure 11. General structure of Sulphanomid

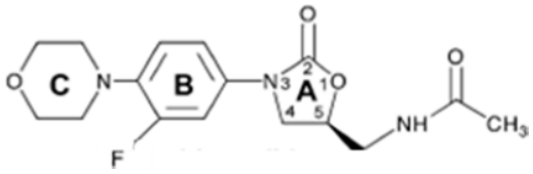
Glycopeptides. Glycopeptides are made of a cyclic peptide of 7 amino acids, to which are bound 2 sugars (Kang and Park, 2015). The formation of 5 hydrogen bonds with the drugs peptidic backbone leads to attaching the antibiotic to its target. Sometimes, an extra sugar and/or chlorine is added during synthesis to the to the backbone of this antibiotic. These supplementary parts are recorded to bind with better efficiency to the target (Walsh & Wencewicz, 2016).



(Adapted from Yim et al., *Frontiers in microbiology*, 2016)

Figure 12. Structures of Glycopeptides (Telcoplanin and vancomycin)

Oxazolidinones. Oxazolidinones have been only recently approved. They are synthetic antibiotics. The first member of this group to be synthesized is Linezolid (Fig.13) (Bozdogan & Appelbaum, 2004). They are described to affect with the synthesis of protein, although the mechanism is yet to be properly explained. They constitute the drug of choice when dealing with surgical infections since this class can penetrate and accumulate easily in the tissue (Bozdogan & ppelbaum, 2004).



(Adapted from Walsh & Wencewicz, *American Society for Microbiology*, 2016)

Figure 13. structures of Linezolid

1.4 Antibiotics and Animal Farming:

In the 1950s, scientists had a breakthrough discovering that animals given low doses of antibiotics are healthier, live longer and grow faster. This helped farmers all around the world in boosting the productivity of dairy cattle and providing them to the consumer at lower costs (Chan-Yuan *et al.,* 2018). Growth promoter antibiotics were licensed by the FDA in 1951 and became a routine part of the business of farming. Used in small doses, these were used for growth promotion to make animals put muscle mass more quickly and less expensively. Then, these doses were raised when it was found that routinely giving antibiotics to animals could prevent them from catching diseases, such as mastitis and rickettsia. The two previous discoveries "growth promoters and preventive dosing" created the meat system that we have today: a very intensive production of animals in relatively small constrained spaces. Moreover, antibiotics are among the most frequently applied antimicrobials in animals meant for food production (Raza & Kim, 2018). Commonly used antimicrobials in dairy cattle fall into five major classes: tetracyclines, sulfonamides, aminoglycosides, macrolides, and β-lactams (Raza & Kim, 2018).

A survey done by collaborators from the Lebanese University on a convenient sample of vets observed that the most commonly used antibiotics in Lebanon for cows are gentamicin, enrofloxacin, TYL and OTC.

OTC and TYL belong to Tetracycline and Macrolide families, respectively. According to FDA, regarding sales of antimicrobial agents for food-producing species, Tetracyclines and Macrolides are third and fourth most relevant classes of antibiotics used in animal farming behind penicillin and sulfonamide (FDA, 2014).

OTCs work by meddling with the bacteria's capability of essential protein production, leading to the inhibition of cell growth. In several countries, OTCs are given to animals as a growth promoter and supplemented at sub-therapeutic amounts to the feed, even though this application for antibiotics was banned in Europe since 2006. The Codex Alimentarius (2015) set the MRL of OTC in milk at 100 mg/kg (Cabizza *et al.*, 2017; Tian *et al.*, 2017). Side effects of using Tetracyclines (including OTC) include gastrointestinal disturbance, photosensitivity, and hepatotoxicity. Furthermore, they have a strong affinity to Calcium and can accumulate in developing teeth and bones leading to discoloration of teeth and inhibition of bone growth (Cabizza *et al.*, 2017).

On the other hand, TYL has a bacteriostatic activity against most organisms. It inhibits the protein elongation process and is usually applied in veterinary medicine showing in vitro activity against a broad range of microorganisms. Maximum Residue Limit (MRL) of TYL is 50 µg/kg (50 ppb) in milk. Most common side effects include vomiting, nausea, and diarrhea (Chan-Yuan *et al.*, 2019).

1.5 Antibiotic resistance:

Antibiotic resistance owing to frequent antibiotic usage has been feared as early as the discovery of the first antibiotic "penicillin". In 1945, Sir Alexander Fleming cautioned against the use of antibiotics in his Nobel prize acceptance speech "there may come a time

where penicillin can be bought by anyone in the shops. Then, there is a danger of the ignorant man may easily underdose himself, and by exposing his microbes to non-lethal levels quantities of the drug, making them resistant". Antibiotic resistance happens when bacteria develops the capability to beat the antimicrobial drugs designed to kill them. This happens when bacteria develops diverse genetic strategies, such as mutation, so that drugs would not be able to recognize their targets; production of damaging enzymes to deactivate antibiotics; driving antibiotics out of the cell; generating a "biofilm" so that drugs are not able reach them; and producing bypasses so they are able to work without the enzymes targeted by antimicrobials (Penesyan *et al.,* 2015). It is hard to treat, and at times impossible, infections caused by antibiotic-resistant bacteria. In most cases, this would lead to a prolonged hospital stay, and expensive and toxic substitutes. Today, nearly all-important bacterial infections all over the world are developing resistance to antibiotics. Antibiotic resistance has been dubbed one of the world's greatest pressing public health problems (FDA, 2014). The use of antibiotics is directly related to antibiotic resistance: the larger the amount of antibiotics consumed, the bigger the risk of antibiotic-resistant populations emerging (Doane *et al*., (2014); Groot *et al.,* (2016); WHO (2012)). Each year in USA, no less than 2.8 million people develop an infection that is resistant to antibiotics, and at least 35,000 people die (CDC 2019).

1.6 Antibiotic resistance and antibiotic residues in milk:

People in any stage of life can be affected by antibiotic resistance. In addition, it is placed as one of the world's highly pressing public health problems (FDA 2018). Antibiotic usage has a clear link with antibiotic usage in animal farming (Doane *et al.,* 2014). As reported

by The US Centers for Disease Control and Prevention (CDC), the prevalent use of antimicrobial drugs in agricultural sector has led to an amplified antibiotic resistance in humans, thereby reducing the effectiveness of antimicrobial drugs for treating human disease (Redding *et al.,* 2016). The administration of these drugs to animals can be orally or through injections, or topically. All these ways of administration could lead to the delivery of antibiotics into the milk (Raza & Kim 2018). Likewise, when cows undergo a prolonged treatment, significant amounts of antimicrobial drugs can be anticipated to be released with the milk (Raza & Kim 2018). There is proof of a global growth in antibiotic resistant bacteria owing to the widespread and improper use of antimicrobial drugs, and their application in animal production, together with dairy farming, as a contributor (Krömker & Leimbach, 2017). Two commonly spread bacteria through food (salmonella and campylobacter) lead to more than 400 000 Americans sick each year with antibiotic resistant infections (CDC, 2013). The issue concerning antibiotic usage in animal production is a global issue and is not only limited to developing countries (Groot *et al.,* 2017).

1.7 Effect of processing:

There are a lot of parameters influencing the degradation of antibiotics such as the food matrix, methods of thermal processing and presence of food additives (Tian *et al.,* 2017). Though the precise impact is still unclear, the food matrix has been proven to indeed influence the degradation of antibiotic residues. There lies a high importance in characterizing and understanding how antibiotics are partitioned in milk and dairy products. This is to project the consumers' potential exposure levels. Doing so by

understanding the drivers of the distribution and concentration of antibiotic residues amongst milk fractions such as lipophilicity, thermal effect, cheese making, and food matrix will enhance and clarify the potential risk assessment for human exposure. Unfortunately, there are a limited number of studies looking at the effect of processing on antibiotic residues in milk and dairy products (Shappell *et al*., 2017, Gajda *et al*., 2017 and Cabizza *et al*., 2016).

Lipophilicity and aqueous distribution. One study looking at the distribution of several antibiotics (OTC, penicillin and erythromycin) between whey, curd, and protein from raw bovine milk, found that it is related strongly to lipophilicity with OTC being the least hydrophobic between the three drugs, having 13% of OTC entrained in the curd (Shappell *et al.,* 2017). Similarly, when looking at the transfer of OTC from bovine spiked milk to cheeses and whey, a study reported that it was mainly recovered in whey and in skimmed milk rather than whole milk due to its high-level hydrophilicity. In conjunction, a study evaluating the distribution of OTC among milk fat and skim milk fractions of cow milk found that most of the drug was distributed into the skimmed milk (more than 90%). Gajda *et al*. (2017) looked at the transfer of tetracyclines (at the level of MRL) from spiked milk to dairy product and reported a non-significant difference in antibiotic concentration between skimmed milk and regular milk (recoveries in excess of 81%). Furthermore, a study analyzed fortified milk samples at 2 time the MRL, looking at the effect of skimming at 4°C for 6 h. For most β-lactams, Macrolides, and Sulphonamides, recovery in skimmed milk was higher than 85%. As for Tetracyclines and quinolones, they were more significantly lost through skimming, but were still recovered with efficiencies between 55% to 80% (Pellicciotti *et al.,* 2016).

Thermal processing. It has been demonstrated that thermal processing reduces antibiotic residue concentration. A review of literature regarding thermal treatments and antibiotic degradation concluded that thermal degradation of antibiotics is temperature-dependent, and in certain temperatures, sustained heating time induced additional degradation, with b-lactams and tetracyclines being most heat-sensitive between the classes, and levamisole being most heat-stable (Tian *et al*., 2017). A study looking at the transfer of TCs (at the level of MRL) from spiked milk to dairy product found no significant effect of low temperature long time pasteurization (the percentage degradation for TCs were below 19% from the pasteurization procedure (63°C for 30 min)) (Gajda *et al*., 2017). The impact on TCs of holder pasteurization reported by Gajda et al. (2017) demonstrated that individual drugs within TC family can differ in regard to their thermostability. They found a similar reduction for OTC (10%) and TC (11%) (Doxycycline (DC) was the most heat resistant with a decrease of 6%). Likewise, a reduction below 20% for OTC, TC and DC after pasteurization was reported by Pellicciotti *et al*. (2016); and a 5.7% reduction for TC and 15.3% for OTC as revealed by Kellnerova *et al*. (2014) when looking at the thermostability of these drugs in milk. As for macrolides, the studies looking at their stability in the milk matrix under different heat treatments are scarce. Zorraquino *et al*. (2011) tested the effect of pasteurization under different conditions on erythromycin, spiramycin and TYL in the milk matrix. They looked at the effect on the antimicrobial activity. The results of this study indicated that processing the milk at 120 °C for 20 min produced an inactivation of 93% for erythromycin, 64% for spiramycin and 51% for TYL. Alternatively, treatment at 140 °C for 10 s resulted in lower percentages (30% erythromycin, 35% spiramycin and 12% TYL). The lowest reduction of antimicrobial activity was obtained by treatment at 60 °C for 30 min.

Cheese making. The obtained results from a study by Gajda *et al.* (2017) demonstrated that in the making of milk derivatives, there is a high level of transfer of antibiotics from milk to these products. For example, the curd and cheese had the highest concentrations (ranging between 320–482 μg/kg and 280–561 μg/kg, respectively) (Gajda et al., 2017). As previously mentioned, Hakk et al. (2016) studied the distribution of OTC among milk fat and skim milk fractions of cow milk. They found that the majority of the drug was distributed into the skimmed milk due to its high hydrophilicity. Hence, finding the greatest levels of this particle in the fractions of whey was expected in a study looking at the transfer of OTC to whey and cheese. Despite that, the results of this study revealed that levels of OTC in 1-day old cheese (3.8 μg/kg), was analogous with those of fat (5.2 μg/kg) and protein (4.6 μg/kg). These results proposed an interaction between the casein matrix of the curd and OTC. In support of this hypothesis, it is known, in fact, that OTC binds to the animal proteins and presents an elevated affinity for Ca^{2+} and Mg^{2+} (Cabizza *et al.,* 2016). This was supported by Samanidou *et al.* (2007) where they studied the relationship between calcium and the degradation of OTC and discovered that calcium causes a slower rate of hydrolysis of OTC (since as aforementioned the antibiotic family of tetracyclines are known to bind strongly to calcium ions). However, studies to date have yet to look at the effect of the food matrix effect on the degradation regarding TCs as it remains unclear. However, there is a scarcity of evidence regarding the effect of milk processing into milk derivatives and the effect it has on the present antibiotics in milk.

1.8 Quantification techniques for antibiotic residues in milk and dairy products:

Extremely complex instrumental methods are needed to analyze qualitatively and quantitatively all antibiotic residues in milk and dairy products.

There are various established analytical methods for the detection of antibiotics in milk and dairy products. There are two classifications for the techniques of analysis: direct methods of analysis and methods involved in extraction/clean-up. Previous methods comprise enzyme-linked immune sorbent assay (ELISA), the Delvo test, and other screening tests, whereas the latter ones commonly depend on techniques of chromatography (Raza & Kim 2018).

Screening methods:

With the lack in structural information and simplicity, bioassays demonstrate a cross reactivity that is significant for structural analogs whilst being only able to deliver measurements that are semi-quantitative of antimicrobial residues (Cullor *et al.*, 1992). Along these lines, comparing these positive results of screening to those of a more selective, reliable, and sensitive physiochemical techniques is crucial. In this regard, screening results that are progressively solid can be offered by chromatographic strategies (Shephard *et al*., 2005).

Extraction and cleanup process:

The detection of antibiotics simultaneously in milk and dairy products is a difficult procedure due to the milk's intricate nature. The milk matrix needs two steps: extraction and clean-up. There are many diverse extraction platforms used for the extraction of analytes (e.g., liquid-liquid extraction (LLE), solid phase extraction (SPE), solid-phase

microextraction (SPME), pressurized liquid extraction (PLE), and matrix solid-phase dispersion solid phase extraction (MMISPE)) (Raza & Kim 2018).

Chromatographic methods:

A scientific review looking at the quantification techniques for important environmental contaminants in milk and dairy products stated that the quantification of antibiotic residues mostly relies on chromatographic methods such as LC-MS and high performance liquid chromatography (HPLC) (Raza & Kim 2018). HPLC has been used broadly in the quantification of antibiotics in dairy samples and is well-thought-out as one of the top analytical methods regarding performance, efficiency, detectability, and sensitivity. However, the combination of mass spectrometry with HPLC gives more precision, higher accuracy, and enhanced detectability. This method appeared as a sensitive means for detecting and determining residual amounts of antibiotic residues in milk and milk products (Raza & Kim 2018). As such, when aiming to detect antibiotic residues in milk and dairy products, MS or MS/MS is considered the gold standard method as it further increases sensitivity and detectability (Raza & Kim 2018).

1.9 Gaps in the literature:

The Annex to Commission Regulation (EU) No. 37/2010 lists the MRL for pharmacologically active substances in foodstuffs of animal origin, including milk. However, at present, milk derivatives have no limits, and this gap in the regulations might complicate both the decision-making on residues by the official control entities, as well as the international trade agreements. The existing literature regarding drug residue transfer from milk to milk-derivatives (e.g., yogurts, cheese, etc.), and their distribution

among the attained fractions (e.g., whey, curd) is very limited. So far, only a few studies have looked at the relation between antibiotic residues in milk and the dairy processing effects on them (Cabizza *et al.* 2017). The U.S. FDA published a risk-assessment entitled "Multicriteria-Based Ranking Model for Risk Management of Animal Drug Residues in Milk and Milk Products" data gaps, including the lack of drug residue distribution data in milk products, were identified. This information, describing partitioning of animal drugs in processed milk and its byproducts, is necessary to ascertain the potential for human exposure (FDA 2015). The complexity of assessing human exposures to drug residues from the products of one "liquid of biological origin "cow milk is evident when considering the multitude of products, including ice cream, yogurt, sour cream, various cheeses, whey protein supplements, and more than 35 others, derived from milk or whey. Understanding the factors driving the distribution and/or concentrating of animal drug residues among milk fractions will allow a better risk assessment for potential human exposure (Shappell *et al.* 2017). For this reason, regulations are in place to set the MRLs of antibiotic residues permitted in food products (Tian *et al*. 2012). But MRLs do not systematically consider the changes occurring during processing, as they only address the quantities of residues present in the raw food commodity. The majority of foods originating are habitually consumed after being cooked or processed. Understanding the impacts of food processing on antibiotic residues is important to assess human exposure, determine MRLs, and assess toxicity (Tian *et al.* 2012).

1.10 Aim of this study:

The scientific literature regarding the transfer of antibiotics from raw milk to milk products are scarce and inadequate. Also, this transfer is directly related to the processes involved in the dairy products making. Thus, investigating the destruction patterns of antibiotics during processing is of prime importance especially among Middle Eastern Cheeses, since these have processes that are particular to them. In other words, the objective of our study is to examine the transfer of OTC and TYL from milk to different Middle Eastern dairy products. For this, the effect of heating, rennet addition, pressing, acidification, skimming and salting was assessed as well. Since LIBNOR does not have any standard when it comes to antibiotic residues in Middle Eastern cheeses, our work will help them in understanding the patterns of destruction and partitioning in locally consumed cheeses, and thus, in developing MRLs.

Chapter 2

Materials and methods

Bovine milk is the most commonly used in the Middle Eastern area, especially that it is available all year long (Maitah & Smutka, 2013 and Merdji *et al*., 2015). We aimed at studying the effect of different processing steps of bovine milk into most commonly consumed Middle Eastern cheeses on TYL and OTC.

2.1. Choice of the Antibiotics:

A short phone-based questionnaire was conducted among a convenient sample of veterinarians employed by dairy farms. It showed that the most commonly used antibiotics for cows in Lebanon are gentamicin, enrofloxacin, TYL and OTC. In our study, OTC and TYL were chosen. These belong to the tetracycline and macrolide families, respectively. Tetracyclines and Macrolides are the third and fourth most important classes of antibiotics used in animal farming regarding sales of antimicrobial agents for food-producing species behind penicillin and sulfonamide (FDA, 2014).

2.2. Choice of The Laboratory:

The Lebanese Agriculture Research Institute- Fanar was chosen to perform the laboratory work since it is officially accredited by the Lebanese government for the detection and quantification of antibiotic residues in food, in addition to the fact that it is ISO17025 certified.

2.3. Chemicals and Reagents:

Oxytetracycline hydrochloride (OTC, purity: 96.7%) and Tylosin Tartrate (purity: 84%) were obtained from Sigma Aldrich (St. Louis, MO, USA). All organic solvents were all high-performance liquid chromatography (HPLC) grade and all chemicals were analytical grade. Formic acid was from Fluka® (Morris Plains, NJ, USA) purchased from Sigma Aldrich (St. Louis, MO, USA). Acetonitrile (ACN) were from J.T. Baker® (Deventer, the Netherlands) purchased from Sigma Aldrich (St. Louis, MO, USA). Syringe 0.22 μm Hydrophilic Polyvinylidene Fluoride (PVDF) Membrane Filters were from Restek (Bellefonte, PA, USA) purchased from Sigma Aldrich (St. Louis, MO, USA). HPLC grade water from Fisher chemical (UK) was purchased from Sigma Aldrich (St. Louis, MO, USA). Analytical reference standards of OTC and TYL used as internal standard and trichloroacetic acid (TCA) were obtained from Sigma-Aldrich Chemical Company (St Louis, MO, USA).

Stock solutions of OTC and TYL were prepared by diluting the pure standard in methanol to a final concentration of 1 g/L and stored in the dark at -20^0C until their use for the analytical procedure.

2.4.Milk inoculation and Cheese Manufacture:

2.5.1. **Milk Inoculation:**

450 kgs of antibiotic-free bovine milk was collected from a flock of healthy and untreated cows from the farm of “Normandie les Fermes”- Zahle, Lebanon. The mean chemical composition of the milk was as follows: total solids, 11.94±0.175% (w/w); protein:

3.35±0.025% (w/w); fat: 3.47±0.029% (w/w); casein, 2.62±0.02% (w/w); and lactose, 4.82±0.09% (w/w).

The collected milk was intentionally inoculated with the chosen antibiotics, whereby 75ml Oxytetravet 30% inj LA (active ingredient: oxytetracycline hydrochloride) and 112.5 ml of Tylokel (active ingredient: Tylosin Tartrate) were added to the initially antibiotic-free milk sample. After which, the spiked milk was divided into three batches (batches A, B and C) for further processing of milk into different cheeses, as illustrated in a scheme of the cheese manufacturing process in table 1.

2.5.2. **Cheese Manufacturing:**

Performed at the R&D Department of Dairy Khoury, a major dairy industry in Lebanon at a pilot scale.

After the intentional inoculation of the milk with TYL and OTC, the milk got parted into three batches, whereby each batch has undergone different processing conditions. In the first batch (A), 100 kgs of the spiked milk has undergone holder (vat) pasteurization (63^{o}C for 30 minutes).

In the second batch (B), 50kg of the inoculated milk was heated at 55^{o}C and centrifuged for skimming. Then, skimmed milk was mixed with another 50 kgs of spiked full fat milk. This is the practice performed in the dairy industry to get better organoleptic quality dairy products. After that, the resultant milk mixture has undergone pasteurization using the holder method (63^{o}C for 30 min).

In the third batch (C), 250 kg of inoculated full fat milk underwent HTST pasteurization (72^{o}C for 15 seconds). The 3 batches of pasteurized milk were then processed to obtain different cheeses.

In the first step (curding), microbial rennet 45 IMCU/L (CHY-MAX® Powder Extra NB, Christian Hansen, Denmark) was added to the milk (at 40^{o}C), then mixed and left to rest for 25 min. Subsequently, the solid curd was collected by filtration, and the liquid whey was separated and collected. The collected curd was drained and shaped in molds to obtain the "Baladi" cheese. Then, Baladi cheese has undergone pressing using a mechanical pressor, and the resulting cheese was the "Akkawi".

Following this, Akkawi cheeses were dropped in boiling water and left until the core temperature of the cheese reached 70^{o}C (for a duration of approximately 35min). The resulting cheese was the Halloum, which was then salted in brining solution (5%) to yield 2% salt Halloum and in brining solution (12%) to yield 6% salt Halloum.

To yield the Double Cream cheese, the whey that was collected in the first step of the cheese production, was heated to 88^{o}C, in the presence of salt and citric acid leading to the clotting of the whey proteins. These proteins were collected to get the Arishe. Then, Arishe was pressed to get Double Dream cheese. The detailed cheese manufacturing and collection is presented in table 1.

Table 1. Cheese manufacturing *

A	B	C
Refrigerated fresh milk in the RAW-VAT-PB2		
0-RAW MILK Sample		
Transfer the milk to 500-VAT-PB2		
Spiked the milk with ATB		
Mix well for 5min		
0-SPIKED MILK Sample		
	Transfer 3 Buckets to Seperator-PC3	
	Heat to 50-60°C	
	Separate the Fat	
	B-SKIMMED RAW Sample	
	B-Cream Sample	
100Kg	50Kg Skimmed - 50Kg	
Heat 63-65°C for 30min		Pasteurize the remaining 225Kg
A-MILK VAT Sample	**B-LOW FAT MILK VAT Sample**	**C-MILK HTST Sample**
Decrease temperature to 40°C		Transfer to 300-VAT-CURD
Add Rennet - Mix and Wait for 20-30min		
Cutting the curd		
Separate the Whey		
A-WHEY Sample	**B-WHEY Sample**	**C-WHEY Sample**
Baladi Production - Draining in Cups		
A-BALADI Sample	**B-BALADI Sample**	**C-BALADI Sample**
Akkawi Production - Pressing in Cloth		
A-AKKAWI Sample	**B-AKKAWI Sample**	**C-AKKAWI Sample**
Halloum Production - Boiling in water/serum		
A-HALLOUM-0 Sample	**B-HALLOUM-0 Sample**	**C-HALLOUM-0 Sample**
Brining (5% and 12%)		
A-HALLOUM-2 Sample	**B-HALLOUM-2 Sample**	**C-HALLOUM-2 Sample**
A-HALLOUM-6 Sample	**B-HALLOUM-6 Sample**	**C-HALLOUM-6 Sample**
Transfer the Whey to Fusore 1		
Heat to reach 88°C		
Add Salt and Citric Acid		

Clotting		
A-SERUM Sample	**B-SERUM Sample**	**C-SERUM Sample**
Double Cream Production - Pressing in Cloth		
A-DOUBLE Sample	**B-DOUBLE Sample**	**C-DOUBLE Sample**

*: as per Dairy Khoury industry

2.6.Milk and Cheese Composition:

Table 2. Fat and moisture content of milk and cheeses

Test	Method	Equipment
Fat	Gerber Method	Gerber tubes & Gerber Centrifuge (Funke Gerber Nova Safety, Germany)
Moisture	Gravimetric method	Moisture analyzer (Sartorius MA35, Germany)

This part was processed in the experimental plant of Dairy Khoury and each analysis was performed in triplicates.

2.7.Analytical method:

2.7.1. Sample Preparation: Liquid-Liquid Extraction:

This method of extraction was developed and verified in the laboratory from the official method (EU) to be able to extract the antibiotics from the sample without the interference of the coextracts. The method is described with what follows (ANSES, 2019).

A 2 ± 0.05 g portion of milk, skimmed milk, whey, serum, Baladi, Akkawi, Halloum 0%, Halloum 2%, Halloum 6% and Double Cream was weighted. Then, for OTC extraction, 200 µl of formic acid 0.1% were added followed by 1 min shacking then left to rest for 10

min in obscurity. This was followed by the addition of 0.5 ml Na2 EDTA and shaking for 1min, after which 8 ml of 5% TCA was added and the mix was shacked for 10min. Next, samples were centrifuged for 10 min at 10 000 x RCF at 4°C. Then, the extracts were filtered through 0.22 µm PVDF filters into amber vials.

As for TYL extraction, similarly, 2g ± 0.05 g of the sample was weighed and 200 µl of formic acid 0.1% added and followed by shaking for 1 min and left in obscurity for 10min. Then, 10 ml of acetonitrile were added to the solution followed by shaking for 5 min. The next step was to centrifuge for 10 min at 10 000 x RCF at 4°C. Then, the extracts were filtered through 0.22 µm PVDF filters into amber vials (ANSES, 2019).

2.7.2. Liquid chromatography – mass spectrometry:

For the analysis of OTC and TYL residues in milk and milk derivatives, a Liquid Chromatograph- Mass Spectrometer (SCHIMADZU LCMS-8045) with HPLC, Nexera X2 LC-30AD Liquid Chromatography, Degassing unit: DGU-20A5R, Communication Bus Mobile CBM-20A and Prominence Column Oven CTO-20AC, was used. The mass spectrometer was operated in the positive ESI ion mode. Nitrogen was used as nebulizer gas, curtain gas and collision gas. The mass spectrometer temperature was 300ºC. The chromatographic separation was performed on a 2.1 *100mm, 3µm column. The elution was performed in a gradient mode.

Table 3: *Separation conditions for OTC and TYL*

OTC / TYL		
Injection	2 µl	
Flux	0.3 ml/min	
Tempreture of the column	40₀C	
Mobile phase	A: water + ammonium acetate (5mM) + 0.1% formic acid	
	B: methanol + ammonium acetate	
Gradient elution		
Time (min)	A%	B%
0	90	10
2	60	40
7	10	90
9	90	10
10	90	10
16	90	10
Total run time (min)	16	

2.7.3. Method validation:

An in-house validation protocol was carried out to establish the performance characteristics of the method, ensuring adequate identification, confirmation, and quantification of OTC and TYL. The method was validated in milk, whey and cheese by the criteria described below.

The selectivity and specificity were assessed by analyzing 3 blank samples from each matrix. The absence of background peaks, above a signal-to-noise ratio of 10, at the retention time of OTC showed that the method was free of endogenous interferences. The validity was determined by analyzing 3 separate samples of milk that were each spiked with OTC at a level of 100 microgram / litre, equivalent to the MRL of OTC in milk. The obtained trueness expressed as the percent of recovery was 105 % for OTC and TYL was 100% for milk. The limit of quantification (LOQ) was defined as the lowest concentration

or mass of the analyte that has been validated with acceptable accuracy, by applying the complete analytical method. The calculated LOQ was 10 mg/ kg for milk.

2.7.4. Linearity:

The linearity was determined through an analytical curve obtained through the LC-MS for each antibiotic. This is through 6 points covering a range of concentration between 100, 300, 500, 600, 800, 1000 and 1500 ppb. The parameter of this calibration curve showed a good linearity, with the correlation coefficient > 0.995. The calibration curves for the two antibiotics are shown in the figures below.

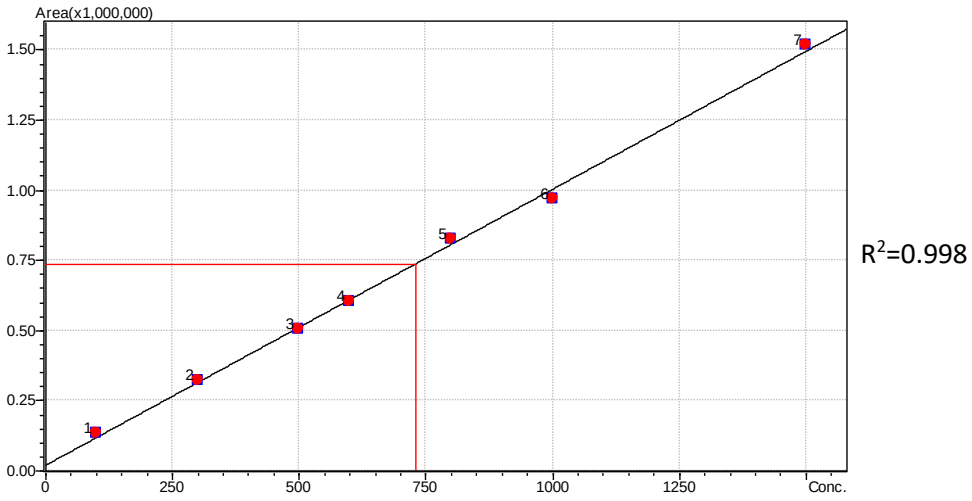
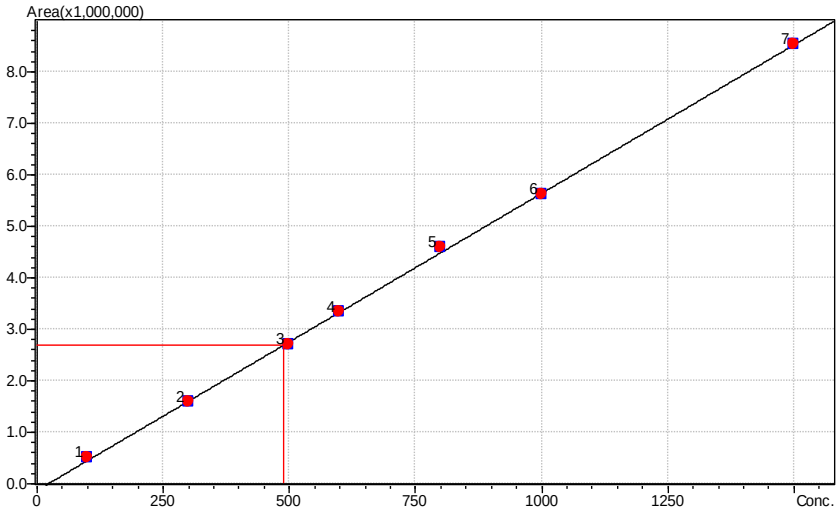


Figure 14: Calibration curve of TYL



R^2=0.999

Figure 15: Calibration curve of OTC

2.8.Statistical analysis:

Analysis was performed using SPSS v25. Descriptive analysis was used to summarize the study variables and to screen for out of range values. Continuous variables were described using mean and standard deviations. Shapiro-wilk was used to assess data normality. Paired t-tests were used to compare the mean differences in OTC and TYL values within groups for the effect of holder heating, curding, pressing, cheese boiling, salting and whey processing. Two-tailed p-values are reported.

Chapter 3

Results

3.1 Moisture and fat composition of the dairy products

To start, it is important to note that the antibiotic inoculation with 8 ppm TYL and 25 ppm OTC had no effect on the cheese making. Tables 4 and 5 represent the fat and moisture content of dairy products

Table 4. Mean fat composition (± standard deviation) of raw and skimmed milk from the different treatments (A, B, C)

Product	Fat	
	Mean	SD
Raw Milk	3.47	0.03
B-SKIMMED RAW MILK	0.08	0.02

Table 5. Mean moisture content (± standard deviation) of Baladi, Akkawi and Halloum cheeses from the three different treatments (A, B, C)

Product	Moisture	
	Mean	SD
C-HALLOUM CHEESE 0%	45.37	0.75
A-HALLOUM CHEESE 2%	53.79	1.17
B-HALLOUM CHEESE 2%	54.38	2.94
C-HALLOUM CHEESE 2%	55.83	0.88
A-HALLOUM CHEESE 6%	46.83	1.17
B-HALLOUM CHEESE 6%	48.34	1.17
C-HALLOUM CHEESE 6%	46.81	1.52
A-BALADI CHEESE	63.11	1.90
B-BALADI CHEESE	63.04	1.32
C-BALADI CHEESE	61.44	1.06
A-AKKAWI CHEESE	56.92	0.26
B-AKKAWI CHEESE	61.24	0.69
C-AKKAWI CHEESE	58.72	0.25

3.2 OTC Distribution in milk components:

To assess whether milk exposure to heat could significantly alter the concentration of the inoculated OTC, we subjected it to different heat treatments that are usually applied in the dairy industry (holder and HTST pasteurization). Figures 16, 17 and 18 show the changes of OTC concentrations through the cheese making processes. Subjecting milk to holder pasteurization decreased significantly ($p=0.015$; table 6) the OTC concentration by 40 to 46 % (table 6). On the other hand, HTST led to an 18% decrease in the concentration of OTC (Fig. 17), but the effect was not significant ($p=0.523$; table 6).

Table 6. Effect of different processes on OTC concentration

	OTC (±SD)			
	Before (ppm)	**After (ppm)**	**Sig**	
Effect of Holder Heating	24.5	14.5	**0.015***	
	26.0	12.0		
	25.25±1.06**	13.25±1.76**		
Effect of HTST Heating	24.5	20.0	0.523	
Effect of Skimming	24.5	20.0	0.411	
Effect of Curding	14.5	31.0	**0.028***	
	12.0	20.0		
	20.0	33.0		
	15.5±4.1**	28.0±7.0**		
Effect of Pressing	31.0	28.0	0.526	
	20.0	22.9		
	33.0	24.0		
	28.0±7.0**	25.0±2.7**		
Effect of Boiling Cheese	28.0	11.4	**0.031***	
	22.9	10.6		
	25.5±3.6**	11.0±0.6**		
Effect of Salting	**0%**	**2%**	**6%**	**Sig**
	11.4	11.5	15.0	0.500
	10.6	9.9	11.0	
		10.0	9.5	
	11.0±0.6**	10.5±0.9**	11.8±2.84**	
	Before (ppm)	**After (ppm)**	**Sig**	
Effect of Whey Processing	3.5	3.0	**0.037***	
	6.0	3.4		
	6.0	3.2		
	5.2±1.4**	3.2±0.2**		

***: Significance level used was 0.05**
****: Mean ± standard deviation**

When it came to skimming, skimmed milk did not significantly (p=0.411) change in concentration from the initial full-fat inoculated milk with only an 18% decrease in

concentration. This was accompanied with a decrease in milk fat concentration from 3.5% in the initial milk to 0.8% in skimmed milk (table 4).

Pasteurization (and skimming in process B) were followed by a curding step to make the Baladi cheese. This process resulted in a significant ($p=0.028$) increase in OTC concentration ranging between 65 and 112% (table 6). Pressing Baladi to make Akkawi cheese had no significant ($p=0.526$) effect on the concentration of OTC (Table 6). This was accompanied by a slight, yet not significant, decrease of 2-6% in moisture content from Baladi to Akkawi. Boiling Akkawi to obtain Halloum cheese caused a significant decrease of OTC (table 6, $p=0.031$) by 54-59%.

On the other hand, salting Halloum at 2 and 6% did not significantly alter OTC concentration. This insignificant change in OTCs concentration in the Halloum cheeses was also accompanied with slight changes in moisture content of Halloum at 0, 2 and 6%. Whilst for whey processing, to make Double Cream, it significantly decreased OTC concentration by 14.7 to 46.3 % (table 6: $p=0.037$).

Our study tracked the OTC transfer from spiked milk to milk derivatives. OTC molecules were non-homogenously distributed between the fractions (whey, Baladi, Akkawi, Halloum and double cream). Mean concentration factors of 1.8 ± 0.3 and 1.7 ± 0.2 from heat treated milk to Baladi (curd) and Akkawi, respectively, were calculated. On the other hand, this factor was calculated as 0.2 for Double Cream cheese made from whey. The highest concentrations among dairy products were reported at a range of 20.0-33.0 ppm and 22.9-28.0 ppm, in Baladi and Akkawi, respectively (fig. 16, 17 and 18).

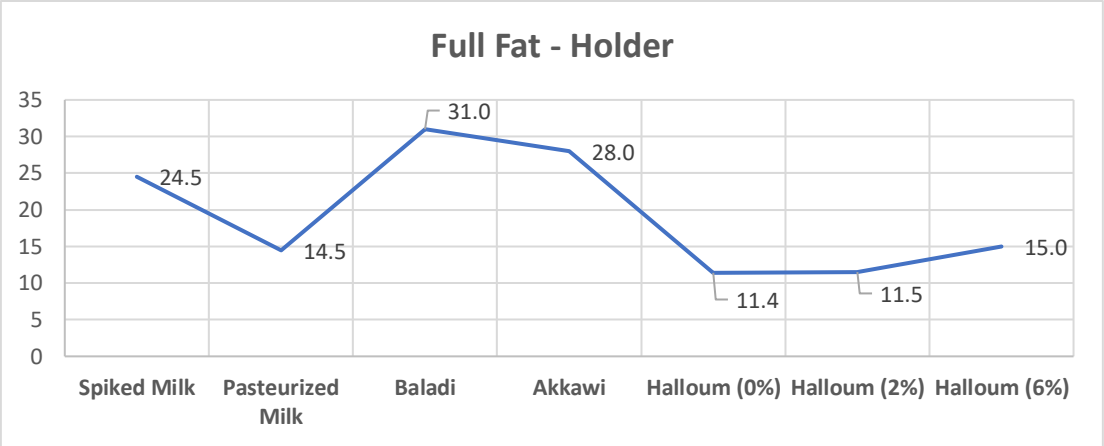


Figure 16: Distribution of OTC (ppm) in milk and cheeses of batch A

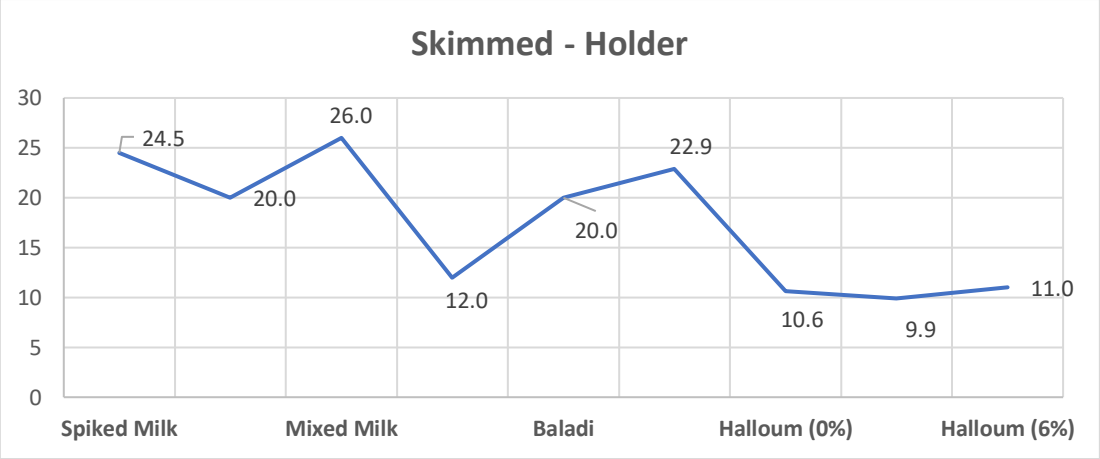


Figure 17: Distribution of OTC (ppm) in milk and cheeses of batch B

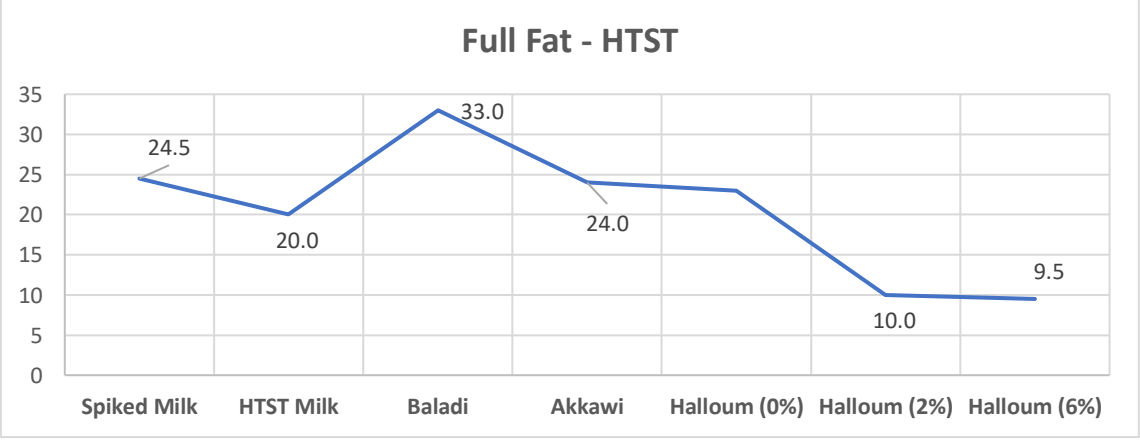


Figure 18: Distribution of OTC (ppm) in milk and cheeses of batch C

The lowest level of OTC was detected in whey and Double Cream cheese (3.5-6.0 and 3.0-3.4 ppm, respectively) (fig. 19).

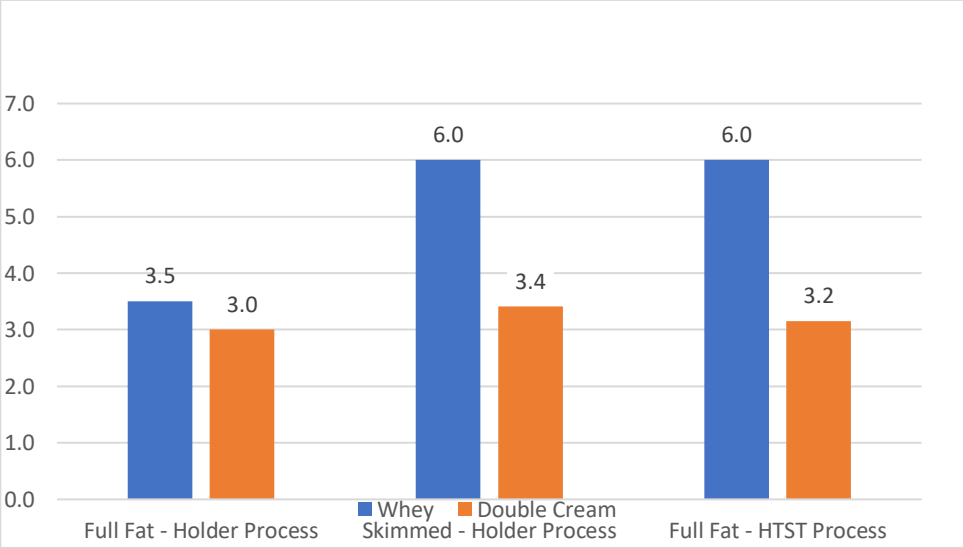


Figure 19: Distribution of OTC (ppm) in whey and Double Cream

3.3 Distribution of TYL in milk components:

As for TYL, figures 20, 21, 22 and 23 show the changes of its concentrations through the cheese making processes. In contrast to OTC, subjecting milk to holder pasteurization insignificantly altered TYL concentration (table 7: p=0.561), and the decrease was 20.5 to 32.0 % (fig. 20 and 21). Whereas HTST, as seen in figure 22, led to a significant decrease of 32% in the concentration of TYL (table 7: p=0.012).

Table 7. Effect of Different processes on TYL concentration.

	TYL (±SD)			
	Before (ppm)	**After (ppm)**	**Sig**	
Effect of Holder Heating	7.8	5.3	0.561	
	3.9	3.1		
	5.85±2.75**	4.2±1.55**		
Effect of HTST Heating	7.8	5.3	**0.012***	
Effect of Skimming	7.8	3.0	**0.015***	
Effect of Curding	3.1	4.4	0.575	
	5.3	5.7		
	4.2±1.6**	5.1±0.9**		
Effect of Pressing	4.4	5.5	0.954	
	5.7	4.7		
	5.1±0.9**	5.1±0.6**		
Effect of Boiling Cheese	5.0	1.6	**0.007***	
	5.5	3.1		
	4.7	2.8		
	5.1±0.4**	2.5±0.8**		
Effect of Salting	**0%**	**2%**	**6%**	**Sig**
	1.6	2.1	2.0	1.000
	3.1	3.2	2.8	
	2.8	2.2	2.6	
	2.5±0.8**	2.5±0.6**	2.5±0.41**	
	Before (ppm)	**After (ppm)**	**Sig**	
Effect of Whey Processing	2.8	8.2	**0.000***	
	2.9	8.6		
	2.5	7.1		
	2.7±0.2**	8.0±0.8**		

***: Significance level used was 0.05**
****: Mean ± standard deviation**

Skimming caused a significant decrease (table 7: p=0.015) by 68.6% in TYL. This was accompanied with a decrease in milk fat concentration from 3.5% in the initial milk to

0.8% in skimmed milk (table 4). Curding resulted in an increase in TYL concentration that was not significant (table 7: p=0.575). Pressing had no significant (table 7: p=0.954) effect on the concentration of TYL. This was accordingly accompanied by a slight, yet not significant, decrease in moisture (table 5). Boiling Akkawi to make Halloum caused a significant decrease of TYL (table 7, p=0.007) by 40-68% (fig. 20, 21 and 22). Salting at 2 and 6% did not significantly alter TYL concentration. This was accompanied with non-significant changes in moisture content of Halloum (table 5).

Conversely to OTC, making of Double Cream lead to a significant increase in TYL concentration (table 7: p=0.000) by 300 % compared to whey (Fig. 22).

These results signify that TYL transfers non-homogenously from spiked milk to milk derivatives (whey, Baladi, Akkawi, Halloum and Double Cream). Mean TYL concentration factors were calculated as 1.25±0.2, between heat treated milk and curd, and between whey and Double Cream, respectively (were of 2.1±0.5).Double Cream had the highest concentration of TYL ranging between 7.1 and 8.6 ppm, being the only cheese that had a level of TYL higher than that of the raw spiked milk (7.8 ppm).

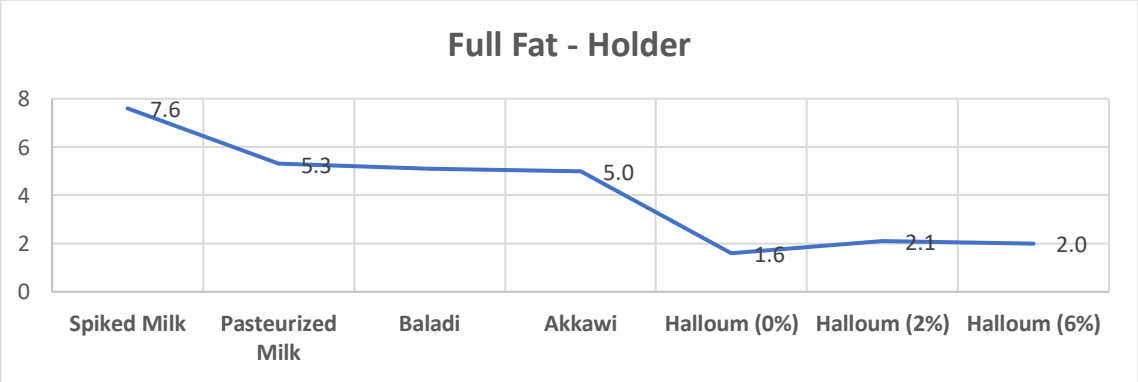


Figure 20: Distribution of TYL (ppm) in milk and cheeses of batch A

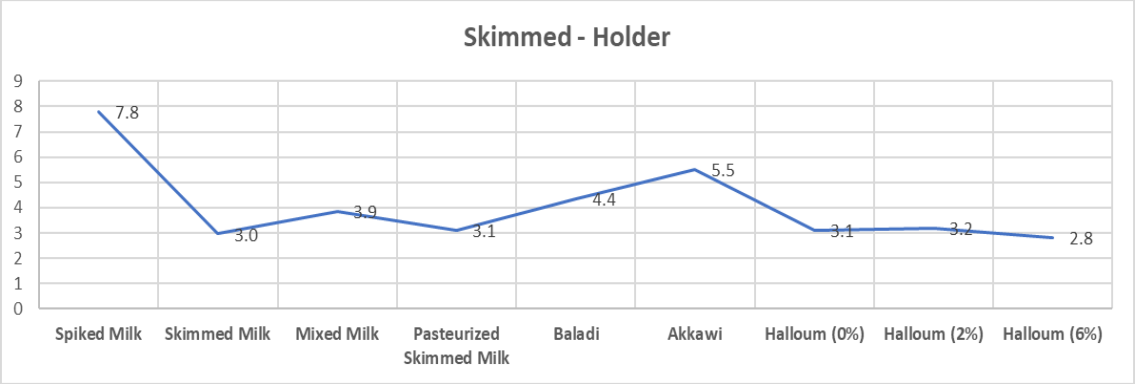


Figure 21: Distribution of TYL in milk and cheeses (in ppm/kg) of batch B

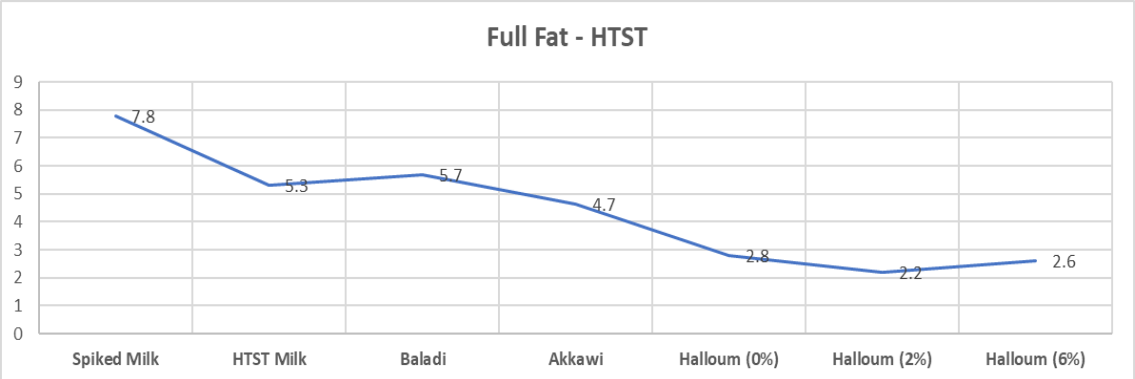


Figure 22: Distribution of TYL (ppm) in milk and cheeses of batch C

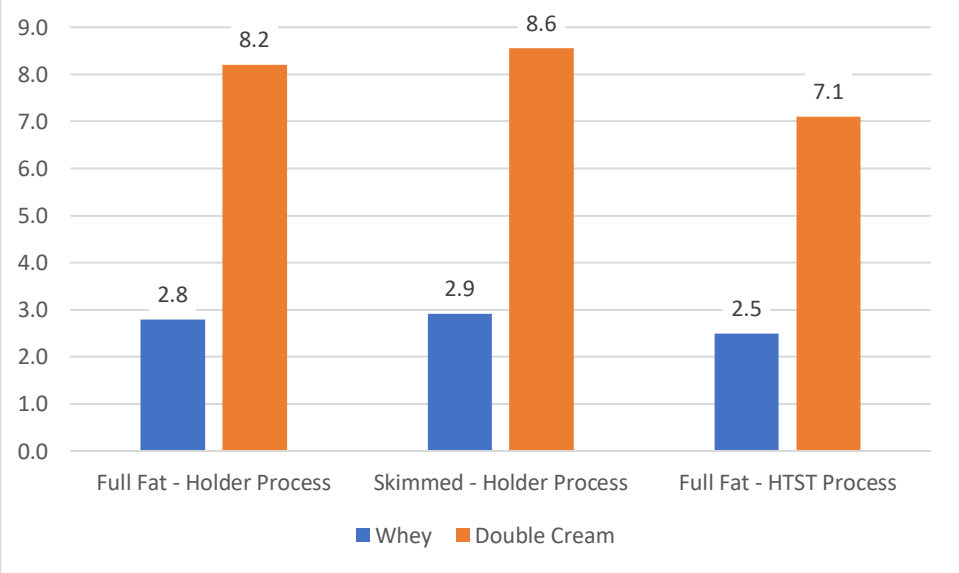


Figure 23: Distribution of TYL (ppm) in whey and Double Cream

Chapter 4

Discussion

4.1. OTC Distribution in dairy products:

There is a strong toxicological importance in drugs residues in milk and their thermal stability. The thermal treatment causes reduction of the concentration of the drug and changes its pharmacologic effects. Many studies reported results addressing the effect of heat processing on antibiotic residues, including OTC with a destruction rate ranging between 25 and 100% (Du *et al*., 1997, Hsieh *et al*., 2011, Ibrahim and Moats, 1994, Kitts *et al*., 1992, Nguyen et al., 2015, O'Brien et al., 1981 and Tian *et al*. 2017). However, only few studies addressed this effect in the milk matrix (Gajda *et al*. 2017and Kellnerova *et al*., 2014).

Our study reported a significant effect of low-temperature long-time holder pasteurization on OTC ($p=0.015$), with mean degradation level of 40% for full-fat milk and 46% for the low-fat milk. Other studies looking at the effect of holder pasteurization on OTC in milk did not report a significant effect with degradation levels of 10% as reported by Gajda *et al*. (2017) and 15.3% as reported by Kellnerova *et al.*, (2014). This could be attributed to the differences in experimental set-ups since our study was done in an industrial setting. However, HTST treatment decreased the concentration of OTC only by 18%, which was not significant ($p=0.523$). No study in the literature reported the effect of HTST on OTC.

Our results suggest sensitivity to thermal process time rather than temperature when it comes to OTC degradation.

When observing the distribution of OTC, the skimming effect was not significant (p= 0.411). OTC was mainly recovered in skimmed milk due to its high hydrophilicity this was similarly reported by Hakk *et al*. (2016). Curding caused a significant increase in OTC concentration from heat treated milk to Baladi (p=0.028). OTC from the initial spiked milk increased by 1.4 folds. This increase of OTC from milk to curd (Baladi) was similarly reported by Cabbiza *et al.* (2017) (by almost 4 folds), Gajda *et al.* (2018) (by 3-5 folds) and Shappell *et al.* (2017) (by almost 2 folds). It is worthy to note that the inoculation level in these aforementioned studies was 100 ppb while it was 25 ppm in our study. This shows that different studies reported different levels of increase in OTCs levels from milk to cheeses made from the curd (showing an increase by almost 4 folds).

Our results propose a high affinity and interaction between OTC and the casein part of the milk. This goes in line with what Cabbiza *et al.* (2017) reported in terms of the OTC's binding to animal proteins and its very high affinity to Ca^{2+} and Mg^{2+}. However, different studies reported different increase levels in the concentration from milk to cheeses. It is unclear yet the reason for the inconsistency across processors in this regard, but it may reflect different methodologies. Note that our study addressed the specifics of the Dairy industry, while none of the other studies did. This would additionally explain the difference in the results between studies.

In relation to the strong affinity of OTC for binding animal proteins, 80% of milk proteins are casein and thus, they are mainly present in the curd fraction (Wal, 2012), and conceivably, OTC becomes bound to and tracks along. However, whey contains 20% of

the milk protein justifying why OTC levels were relatively low in the whey fractions. In addition, there was a significant decrease from whey to double cream cheese ($p=0.037$).

It should be highlighted that MRL level given to OTC is meant to ensure zero effect on human health from consumption of milk or its derived products where this compound is present. Since OTC was found to be concentrated in Baladi and Akkawi, special attention must be given when it comes to the dietary exposure of OTC from consuming these two products compared to other cheeses like Halloum and Double Cream.

The effect of boiling Akkawi to obtain Halloum reported in our study was in accordance with other studies in the literature where boiling matrixes containing OTC caused significant destruction of this antibiotic. So, in the case of Halloum the level of OTC, became well below the initial inoculation value, making it safer to consume.

4.2. TYL Distribution in dairy products:

Studies looking at the effect of heat treatment on TYL are scarce. Only one study looked at different heat treatments and their effect on TYL in milk using antimicrobial essays. This study found a 51% decrease in TYL concentration after heating the milk at 120 °C for 20 min, 21% decrease when heated at 140 °C for 10 s and an even lower reduction when exposed to holder pasteurization (Zorraquino *et al.*, 2011).

Similarly, our results convey an insignificant effect of holder pasteurization on the destruction of TYL. The degradation levels ranged from 20.5% for the mixed milk and 32.0% for the full-fat milk. Conversely, the HTST treatment decreased the TYL levels by 32 % showing a significant effect ($p=0.012$). Moreover, this shows that the time temperature combination has a different effect on different antibiotics, when comparing the results of OTC to TYL.

As for the difference in terms of outcome between studies, as mentioned with OTC, could be ascribed to the variances in experimental set-ups and the use of different antibiotic detection methods.

Skimming caused a significant decrease in TYLs concentration (p= 0.015). TYL was recovered mostly in the cream of the milk, indicating a high affinity on fat that exceeds the hydrophilic nature of TYL. Note that no previous studies looked at the effect of skimming on TYL.

No reported study in the literature addressed the effect of cheese making on TYL. However, in contrast to OTC, curding step in our study caused a non-significant increase in TYL concentration from milk to Baladi (curd). This could be attributed to the hydrophilic nature of TYL and to its low affinity to casein proteins. This goes in line with Avci & Elmas, (2014), Ziv & Sulman (1973) and Gingerich *et al.* (1977), who reported that TYL was found to bind to casein at a rate of 15% only. Our finding can be explained by the fact that, when looking at yields, the mass of Baladi consists of only 10% of that of whey predicting a slight increase in TYL concentration in Baladi (fig. 20, 21 and 22).

A significant (p=0.000) increase in the TYL concentration was observed when processing whey to Double cream. This might suggest that contrary to OTC, TYL might have a high affinity to whey proteins with the concentration increasing by almost 3 folds from whey to Double Cream.

Subsequently TYL was concentrated in double Cream, making this product less safe when it comes to TYL exposure from its consumption. This points out the need for regulations regarding cheeses and antibiotic levels and enforcing safety limits. Moreover, our results indicate that cheeses produced from the whey fraction might be of higher risk owing to

TYL levels, while those made from the curd such as Baladi cheese seem to be safer to consume in this regard.

The effect of boiling Akkawi to obtain Halloum reported in our study was significant ($p=0.007$). This process involves reaching a core temperature of 70^{o}C (for a duration of approximately 35min), this is like the temperature conditions of HTST pasteurization for a longer period of time. As previously mentioned, studies in the literature and our results support that these conditions cause a significant level of destruction of TYL. So, in the case of Halloum making, the significant decrease in the levels of TYL makes it safer to consume.

4.3. Limitations:

As for the limitations of our study, there were some outliers in the results. This maybe, in our opinion, due to the contribution of the uncertainty of the adopted analytical method and in order to address this in future studies, analysis should be done in duplicates or triplicates, we did this for most samples, but in some cases this was not possible (due to lack of time and the lockdown for the COVID-19). Moreover, we inoculated OTC and TYL at a level that is well above the MRLs for these antibiotics. As for future considerations, a study done with an inoculation level at the MRL would help to check if there is a dose-dependency with regards to OTC and the studied parameters. In addition, the inclusion of in vivo distribution of the drugs that may differ from in vitro laboratory equilibrations and metabolites or degradation products should be considered.

Chapter 5

Conclusion

Looking at antibiotic residues in milk and milk products is shown to be an important aspect from a health and processing point of view. Our first-of-its-kind study on Middle Eastern cheeses proved that inoculating the milk with OTC and TYL, even at concentrations above MRL, did not cause any alteration in the cheese making. Furthermore, OTC levels were significantly affected by holder pasteurization, but not with HTST. As for TYL, HTST had a significant effect while holder pasteurization did not cause significant destruction. As for milks derivatives, OTC is transferred from spiked milk to milk products, especially Baladi and Akkawi, making them higher risk products for this antibiotic compared to Halloum and Double Cream. In contrast, Double Cream had a high level of TYL transfer, while the other cheeses did not. Hence it is important to highlight that people who consume excessively these products, in which OTC and TYL might become concentrated, could be exposed to significant amounts of OTC or TYL. As a result, it is essential to employ control methods correctly all through the whole milk production chain to avoid any possible risk that the presence of antibiotics might cause.

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