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Effects of Different Processing Methods on the Antibiotic Enrofloxacin Residues Occurrence in Middle Eastern Dairy Products

By

Rita Haddad

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THESIS APPROVAL FORM

Student Name: Rita Haddad I.D. #: 201201997

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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: 07 / 09 / 20
(Day / Month / Year)

Committee Member's Name: Chadi Hoari

Signature: [redacted] Date: 07 / 09 / 20
(Day / Month / Year)

Committee Member's Name: Mireille Serhan

Signature: [redacted] Date: 07 / 09 / 20
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Effects of Different Processing Methods on the Antibiotic Enrofloxacin Residues Occurrence in Middle Eastern Dairy Products

Rita Haddad

ABSTRACT

Antibiotics are used to treat or prevent certain diseases caused by infectious agents. Over the past years, Lebanon has witnessed an increasing consumption of antibiotics through medications and food supply. There is a well-reported link between using antibiotics in farming and developing antibiotic resistance, which leads to subsequent human antibiotic treatment failure. Our first-of-its-kind study in the region aims to determine the effects of different dairy processing unit operations (pasteurization, skimming, curding, pressing, boiling/acidifying, and salting) on Enrofloxacin (ENF), a widely used antibiotic in Lebanon. For this, full fat and skimmed milk were inoculated with ENF, and processed into pasteurized milk along with commonly consumed Middle Eastern products (*Baladi*, *Double Crème*, *Halloum* and *Akkawi*) in a major dairy industry. Liquid Chromatography Mass Spectrometry (LCMS), the gold standard method, was used to quantify ENF residues. Results showed that pasteurization (Holder and High Temperature Short Time) significantly decreased ENF concentration. A significant increase in ENF by 3.5 folds was reported upon curding milk into *Baladi*, and by 1.9 folds upon acidifying/boiling the whey into *Double Crème*. Pressing *Baladi* to produce *Akkawi* did not have any significant effect on ENF concentration, while boiling *Akkawi* to produce *Halloum* decreased it significantly. Salting *Halloum* in brine (12%) did not have any significant effect. Our results will help the local authorities to set MRLs of ENF in dairy products. They will also guide the dairy industries to decide on which product to process the raw milk to whenever they receive milk contaminated with ENF to ensure the safety of their products.

Keywords: Enrofloxacin, Antibiotics, Middle Eastern Cheeses, LCMS, Lebanon

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LIST OF ABBREVIATIONS

AB: Antibiotic

CDC: Center for Disease Control

ENF: Enrofloxacin

HTST: High Temperature Short Time

HPLC: High Performance Liquid Chromatography

LCMS: Liquid Chromatography Mass Spectrometry

LLE: Liquid-Liquid Extraction

MRL: Maximum Residue Limit

PVDF: Polyvinlidene Fluoride

SPE: Solid Phase Extraction

Chapter One

LITERATURE REVIEW

1.1 BACKGROUND:

Antibiotics were first introduced in the 1940s, and since then they have been used extensively in both the healthcare and veterinary fields (Groot & E. van't Hoooft, 2016), and with this practice emerged an increase in antibiotic resistance (Krömker & Leimbach, 2017). Antibiotics, also known as antibacterials, are administered for the prevention or treatment of diseases caused by infectious agents. There has been an exponential increase in the usage of these antibiotics in the last decades worldwide, where higher consumption was evidenced in developing countries compared to that in developed countries. This increased consumption of antibiotics is directly related to the increase in the world population, whereby the world population is anticipated to reach 9.7 billion in 2050 and it is probable that it would reach at nearly 11 billion people by the year 2100 (UN World Population Projections, 2019). This exponential increase is expected to cause an increase in the demand and production of high-quality animal protein and nutritious food sources for everyone on the planet. Dairy farming is regarded as one of the important processes to satisfy this need, especially in developing countries. According to a report released by the Food and Agriculture Organization with the title "World Agriculture towards 2015/2030 a FAO perspective", the annual consumption of milk and dairy products, between the years 1997/99 and 2030, is expected to increase from 45 kg/ person to reach 66 kg/person in developing countries, and from 212 to 221 kg/person in developed countries (FAO, 2014). According to the Lebanese Dairy Board, the annual dairy production was 62,000 metric tons in 2016, whereas its consumption is 14 liters of milk (fresh and powdered) and 24 kg of cheese per capita/year (Blom Invest, 2016). A study performed to evaluate the food consumption pattern of adults living in Beirut, Lebanon, concluded that 10.9% of the daily energy intake of Beirut residents is from milk and dairy products, whereby they tend to consume 243.1 g/ day of milk and dairy products (Nasreddine, Hwalla, Sibai, Hamzé, & Parent-Massin, 2006). In another study that aimed at studying the change in the dietary trends in the

Middle East and North Africa for a period of 46 years, between the years 1961 and 2007, using FAOSTAT database- FAO food balance sheets, the consumption of milk and dairy was found to increase over the years, however, it has not reached the recommendation of 2-3 servings per day (Golzarand et al., 2012). Animal milk is a source of high-quality nutrition to people of different ages due to its rich content of micro- and macronutrients. Lebanon has experienced an increase in the usage of antibiotics not only for humans but also in the veterinary world (Klein et al., 2018). Worldwide, about 50% of all antibiotics produced are used in the veterinary field. Although there is a benefit from using antibiotics, this overconsumption led to the development of multi-resistant microbes consequently resulting in a global crisis (Kabrite, Bou-Mitri, Fares, Hassan, & Boumosleh, 2019)

1.2 ANIMAL MILK AND MILK PRODUCTS:

Milk, a nutritious white liquid, is an important food source of macronutrients like proteins and fat. It is rich in several micronutrients including, calcium, zinc, selenium, riboflavin, magnesium, pantothenic acid (vitamin B5), and cobalamin (vitamin B12) (Haug, Høstmark, & Harstad, 2007b). Milk has a dynamic nature, whereby its composition is affected by several factors including the age and breed of the cattle, its feed and nutrient source, stage of lactation, and health status of the udder (Ontsouka, Bruckmaier, & Blum, 2003). Products obtained and produced from milk vary in nutritional, cultural components, and composition. These products include milk, milk powders, dairy beverages, butter, yogurt, ice cream, frozen desserts, cheese, and cultured dairy. The Middle Eastern area has a variety of traditional white cheeses, including but not limited to *Akkawi, Double Crème, Halloum, Areeshe*. These products form an important source of many nutrients essential for the health and body (Raza & Kim, 2018).

1.2.1 BOVINE MILK AND ITS COMPOSITION:

Bovine milk is the milk produced by cattle. It is composed of amino acids, proteins, lipids, minerals, and vitamins, which are needed for growth and development.

Lipids range from 3.6 to 5.4% and they are emulsified in globules covered with membranes. On the other hand, proteins of the milk mainly casein, occur in colloidal dispersions as casein micelles made up of salts (primarily calcium) and proteins. Lactose and most minerals exist freely in the milk solution. Specific proteins of the milk have a function in the early development of immune response, while other proteins, such as lactoferrin, are involved in the non-immunological defense (Bernabucci et al., 2015). The composition of milk has a dynamic nature and is affected by different climate conditions (Haug, Høstmark, & Harstad, 2007a).

1.2.2 History of Cheesemaking:

Cheesemaking is the process of converting milk, which is an unstable, highly nutritious liquid (raw material) into cheeses that are more stable, flavorsome, and concentrated solid with an extended shelf life. For thousands of years, the process of cheesemaking has been done, and in the majority of time, in the industry of cottage preparation. Towards the end of the 19th century, when industrialization and food processing advanced, cheese manufacture switched into the factory. Since then, there has been a progressive development in that technology especially in the machinery. Nowadays, the dairy industry has developed to include highly automated, large, modern factories hiring minimal staff. Several factors, including cost, increased hygiene, availability of labor, and the need for product consistency and uniformity, have driven this move. Unfortunately, this development has been at the cost of some variety and individuality; therefore, along with the augmented mechanization of the manufacturing facility, there has been a rebirth of many small boutique cheesemakers. The impact of automation and computers on the process of cheesemaking has been dramatic because of replacing all the manual procedures in controlling, programming, data-logging and analysis, by computers, to achieve better uniformity in the production (Legg, Carr, Bennett, & Johnston, 2017).

1.2.3 Middle Eastern Cheeses:

The Middle Eastern Area is known for its wide variety of dairy products, such as *Laban, Labneh, Keshek* as well as a wide variety of cheeses. These cheeses are either ripened cheeses such as *Shankleesh* and *Ricotta*, or brined white cheeses such as *Akkawi*, *Halloumi, Nabulsi, Double Crème* and *Baladi* etc. The preparation of the brined Middle Eastern cheeses occurs through a series of steps. First, the milk is pasteurized, later the rennet is added to separate the milk into whey and curd. The collected curd is molded to produce the *Baladi* cheeses. *Baladi* cheeses are later pressed to obtain the *Akkawi* cheese, which in turn undergoes boiling to collect the *Halloum* cheese. On the other hand, the collected whey undergoes acidification and boiling to obtain the *Arishe* which in turn undergoes pressing to get the *Double Crème*. A scheme of the manufacturing of Middle Eastern Cheeses is presented in Figure 1.

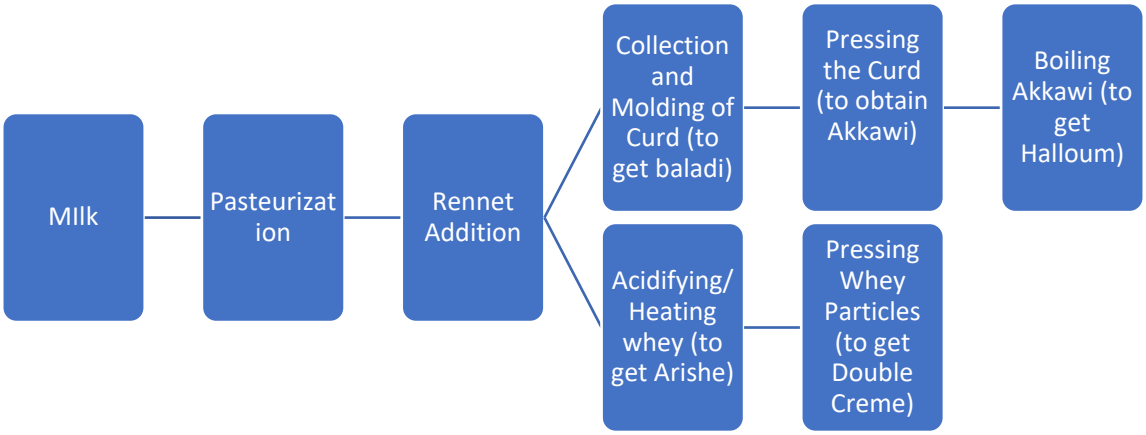

FIGURE 1: SCHEME OF THE MANUFACTURING OF MIDDLE EASTERN CHEESES

1.3 ANTIBIOTICS/ ANTIMICROBIAL DRUGS:

1.3.1 DEFINITION OF ANTIBIOTICS:

Antibiotics are microbiologically produced compounds used for the prevention or treatment of certain diseases caused by infectious agents in both animals and humans.

Antibiotics used for the treatment of humans may belong to the same general class or may have the same mode of action as those used for animals (Joshi, 2002). The administration of antibacterials in animals has many objectives, including therapeutic application for disease treatment, prophylactic utilization to prevent infection, and growth promotion to improve feed utilization and production (Barton, 2000).

1.3.2 USE OF ANTIBIOTICS IN ANIMALS

In lactating cows, antibiotics are administered most of the time to treat mastitis, but in some cases, they are used for the treatment or prevention of other diseases (Rama, Lucatello, Benetti, Galina, & Bajraktari, 2017). Unfortunately, many farmers do not abide by the recommendations that are advised by the different regulatory bodies for the proper administration of the right amount of antibiotics and the use of the appropriate antibiotic for the treatment of a specific bacteria (Ayukekbong, Ntemgwa, & Atabe, 2017). Antibiotics are administered to cattle frequently, whereby approximately 80% of all animals that are involved in food production are administered antibiotics either in a certain period of their lives or throughout all their life spans (Lee, Lee, & Ryu, 2001). Antibacterial drugs used in animals might result in the deposition of residues in their proteins meat, milk, and eggs (Nisha, 2008) .

The U.S. Food and Drug Administration (FDA) established a multicriteria-based ranking model that is used for the supervision and control of animal drug residues in milk and milk products. This risk assessment is used as a decision-support tool to help in deciding which animal drug residues must be included in milk testing programs . The FDA selected 54 animal drugs and their different forms for assessment. However, during the preparation of the risk assessment, data gaps were recognized including the scarce availability of data about the dispersal of the residues of drugs in milk products. Information describing the distribution of animal drugs in processed milk and its by-products is essential to determine the potential for human exposure (*Multicriteria-based ranking model for risk management of animal drug residues in milk and milk products; extension of comment period*2015) .

1.3.3 Concerns with the Use of Antibiotics in Veterinary Medicine:

Once antibiotics are administered to the cattle, the drug is transferred to the proteins of the cattle and then gets transferred to its secretions like milk, which would lead to antibiotic resistance. Drug residues modify the processing qualities of raw milk through inhibiting the starter cultures used in the formation of cheese as well as other fermented dairy products such as yogurt (Brady & Katz, 1988). The presence of antimicrobial drug residues in milk may cause allergic reactions in some hypersensitive persons (Dewdney et al., 1991). Also, it may induce populations of bacteria that are resistant and that do not respond to treatments usually administered for human illnesses (Van Dresser & Wilcke, 1989). The presence of antimicrobials in milk can affect the manufacturing of dairy products, decrease flavor and production accompanying with butter production, reduce the cheese curding process, and cause improper ripening of cheeses (Payne, Craigmill, Riviere, & Webb, 2006).

1.3.4 Mode of Action of Antibiotics:

While some antibiotics act by completely killing bacteria, others function by inhibiting the growth of these bacteria. Antibiotics that kill bacteria are classified as being bactericidal, whereas those that inhibit bacterial growth are classified as being bacteriostatic (Walsh, 2003). Although the term, "antibiotic", in general refers to antibacterial antibiotic compounds, it could be differentiated into antifungals, antivirals, and antibacterials reflecting the group of microorganisms they antagonize (Russell, 2004).

1.3.5 Classes of Antibiotics:

Antibiotics are classified based on a variety of differences between the different antibiotics. The most common classification of antibiotics is based on their mode of action (bacteriostatic vs. bactericidal), molecular structures, and spectrum of activity (broad vs. specific) (Schwalbe & Steele-Moore, 2007). Other classifications include their route of administration (oral, injectable, or topical). Antibiotics belonging to the same structural class show similar allergic potential side effects, along with similar patterns of

effectiveness, and toxicity. Some common classes of antibiotics based on molecular or chemical structures include Quinolones, Beta-lactams, Aminoglycosides, Macrolides, Glycopeptides, Sulphonamides, Tetracyclines, and Oxazolidinones. (Gbaguidi-Haore et al., 2013) (Frank & Tacconelli, 2011) (Munteanu, Titoiu, Marty, & Vasilescu, 2018) (Adzitey, 2015). Table 1 shows a summary of the different classes of antibiotics.

TABLE 1: SUMMARY OF THE DIFFERENT CLASSES OF ANTIBIOTICS ALONG WITH THEIR MODE OF ACTION (GBAGUIDI-HAORE ET AL., 2013) TACCONELLI, 2011) (MUNTEANU ET AL., 2018) (ADZITEY, 2015).

Class of Antibiotics	Examples	Bacteriostatic vs. bactericidal	Mode of Action
Beta- Lactam	- Penicillin - Cephalosporins - Monobactams - Carbapenems - Carbacephems	Bactericidal	Inhibition of bacteria cell wall biosynthesis
Aminoglycosides	- Streptomycin - Neomycin - Gentamicin - Tobramycin - Amikacin - Plazomicin - Paromomycin	Bactericidal	Inhibition of synthesis of protein (Translation) by bacteria, causing cell death
Glycopeptides	- Vancomycin - Teicoplanin - Telavancin - Ramoplanin - Decaplanin - Corbomycin - Complestatin - Bleomycin.	Bactericidal	Inhibition of bacteria cell call biosynthesis
Ansamycins	- Rifamycin - Geldanamycin - Naphthomycin	Bactericidal	Inhibition of the synthesis of RNA by bacteria, causing cell death
Streptogramins	- Pristinamycin IIA - Pristinamycin IA	Bactericidal	Inhibition of synthesis of protein (Translation) by

			bacteria, causing cell death
Quinolones	- Ciprofloxacin - Enrofloxacin - Levofloxacin - Trovafloxacin	Bactericidal	Interference with bacteria DNA replication and transcription
Lipopeptides	- Daptomycin - Surfacin	Bactericidal	Disruption of the functions of multiple cell membrane, causing cell death
Chloramphenicol	- Chloramphenicol - Chloromycetin	Bacteriostatic	Inhibition of protein synthesis, preventing growth
Sulfonamides	- Sulfamethoxazole-trimethoprim - Erythromycin - Sulfisoxazole	Bacteriostatic	Prevention of the growth and multiplication of bacteria
Tetracyclines	- Tetracycline - Doxycycline - Oxytetracycline - Lymecycline	Bacteriostatic	Inhibition of protein synthesis by bacteria
Macrolides	- Erythromycin - Azithromycin - Clarithromycin	Bacteriostatic	Inhibition of bacterial protein synthesis
Oxazolidinones	- Linezolid - Posizolid - Tedizolid	Bacteriostatic	Inhibition of bacterial protein synthesis

1.3.5.1 QUINOLONES:

Quinolones are a class of antibiotics that acts through interfering with DNA transcription and replication in bacteria. From the basic molecule, two main groups of compounds were developed. The first is quinolones and the second is naphthyridones. Those two major compounds include enrofloxacin, temafloxacin, cinoxacin, ofloxacin, sparfloxacin, ciprofloxacin, norfloxacin, nalidixic acid, enoxacin, etc. (Domagala, 1994). Since their discovery in the 1960s, the parent structure has undergone several alterations. In general, their structure is made up of two rings, but generations of quinolones that were recently developed and modified to have an extra ring structure, extended their

antimicrobial spectrum of activity to include bacteria, mainly anaerobic bacteria, that were earlier resistant to quinolone. Also, this has improved their potency and enhanced their effectiveness in the treatment of different forms of illnesses. Another benefit was designing generations tailored particularly for animal use only and not for human use (Naeem, Badshah, Muska, Ahmad, & Khan, 2016)

ENROFLOXACIN

Enrofloxacin (ENF) is a lab-made chemotherapeutic agent derived from the class of the fluoroquinolone carboxylic acid derivatives. It is a third-generation fluoroquinolone antibiotic that is used in veterinary medicine only. Synonyms and trade names of enrofloxacin are Bay Vp 2674, Baytril, Endrofloxicin, and enrofloxacin. It possesses antibacterial action against a broad spectrum of both Gram- positive and Gram- negative bacteria. Its mechanism of action is not well understood, but experiments have been supposing that it acts through inhibiting bacterial DNA gyrase (a type-II topoisomerase), and thus preventing DNA supercoiling synthesis. Enrofloxacin is a bactericidal agent. Its bactericidal activity is concentration-dependent, whereby bacterial cell death occurs within 20-30 minutes of antibiotic exposure. It is a lipophilic antibiotic, which means it tends to dissolve in fat (FAO, 1997).

1.4 ANTIBIOTIC RESIDUES:

1.4.1 DEFINITION OF ANTIBIOTIC RESIDUES:

Antibiotic residues are the byproducts of the antibiotics that remain after the cattle gets treated with antibiotics. The most common cause for the administration of antibiotics in dairy cows is mastitis treatment, which is the inflammation of the breast tissue of the cow (Abebe, Hatiya, Abera, Megersa, & Asmare, 2016). For this purpose, antibiotics get administered through the intramammary route, and that is because of its proximity to the infection site and in an aim to consume less antibiotics (Pyörälä, 2009). However, the main reason of the probable occurrence of antibiotic residues in milk is because the given drugs can be easily relocated to it from the mammary gland. In addition, the fermentation of milk for the production of certain types of cheeses could be altered by the presence of

antimicrobials. None the less, the key problem is the effect on human health because the antimicrobials that are administered for humans and on farms are the same in some cases.

1.4.2 Effect of Antibiotic Residues on Health:

From the health perspective, there are two main concerns with antibiotic residues from the food chain reaching the human body. The first one is the development of antibiotic resistance through the migration of bacterial strains that are resistant to bacteria from animals to humans. The second one is the occurrence of concentrations of antibiotics in milk in levels higher than the acceptable limit, resulting in serious adverse effects on the health (Ben et al., 2019) .The consumption of milk containing antimicrobial residues can stimulate drug hypersensitivity reactions, which can be demonstrated as dermal reactions, asthma, or anaphylactic shock. Moreover, these antibiotic residues can cause antibiotic resistance whereby the human body will learn how to fight the antibiotic; thereby, the antibiotic will no longer be efficient for the treatment of some bacterial infections (Paterson, Hoyle, Ochoa, Baker-Austin, & Taylor, 2016). The usage of antimicrobials might cause an increase in the development of antibiotic resistance for pathogenic bacteria, which might be reflected in a global health crisis (Antibiotic resistance.).

1.4.3 International Opinion on Antibiotic Residues

Regulatory authorities worldwide have endorsed maximum residue limits (MRLs) for several veterinary drugs in food to ensure the safety of human food. These MRLs represent the acceptable daily intake (ADI) in addition to other safety factors.

The Annex to Commission Regulation (EU) No. 37/2010 lists the MRL for pharmacologically active substances in foodstuffs of animal origin, including milk, and it states that the MRL for ENF in milk is 100 µg/kg (RÖMPP-Autor, 2010).

In 2017, the joint FAO/WHO Expert Committee on Food Additives and Contaminants (JECFA), published an update of acceptable MRL values for some veterinary drug residues in some food types. However, no updated MRL values of ENF in milk were stated.

1.5 ANTIBIOTIC RESISTANCE AND THE FOOD CHAIN:

For long years, research studying antibiotic resistance have focused mostly on clinically relevant bacterial species and health-care facilities (Landers, Cohen, Wittum, & Larson, 2012). Conversely, it has been well recognized that the antibiotics that are used for livestock have increased the spread of antimicrobial resistance genes or antibiotic-resistant bacteria throughout the food production chain to reach consumers and their surrounding environments. Besides, it is assumed that foodstuff may play a significant part in this phenomenon since ready-to-eat food and raw food may be cross-contaminated with antimicrobial-resistant bacteria during their processing and manufacturing and thus their safety for consumption becomes questionable (Founou, Founou, & Essack, 2016), (Bengtsson-Palme, 2017), (Verraes et al., 2013).

1.6 PROCESSING EFFECTS ON ANTIBIOTIC RESIDUES IN DAIRY INDUSTRY:

1.6.1 EFFECT OF HEAT TREATMENT

In the dairy industry, pasteurization is the procedure of heating milk or milk product in special equipment that are properly designed for that specific purpose. There are two different common pasteurization methods. The first one is Vat Pasteurization or Holder, whereby the temperature is held at 63ºC (145ºF) for 30 mins, and this is usually performed in small-scale, dairy industries. On the other hand, the second pasteurization technique is called High-Temperature Short Time Pasteurization (HTST), through which the temperature of the milk is held at 72ºC (161ºF) for 15 seconds. This technique is used at larger scale dairy industries (International dairy foods association; international dairy foods association names J. david carlin senior vice president of legislative affairs.2014) . Pasteurization causes various modifications in milk during the process of elimination and reduction of microorganisms existing in milk. There will be physico-chemical and/ or enzymatic modifications, such as activation of plasminogen, whey protein denaturation, and inactivation of lipoprotein lipase and acid proteinase. The consequences of the process of pasteurization on properties and characteristics of milk, that are important in the process

of cheesemaking and ripening, are summarized in *Figure 2* which was adopted from (Grappin & Beuvier, 1997):

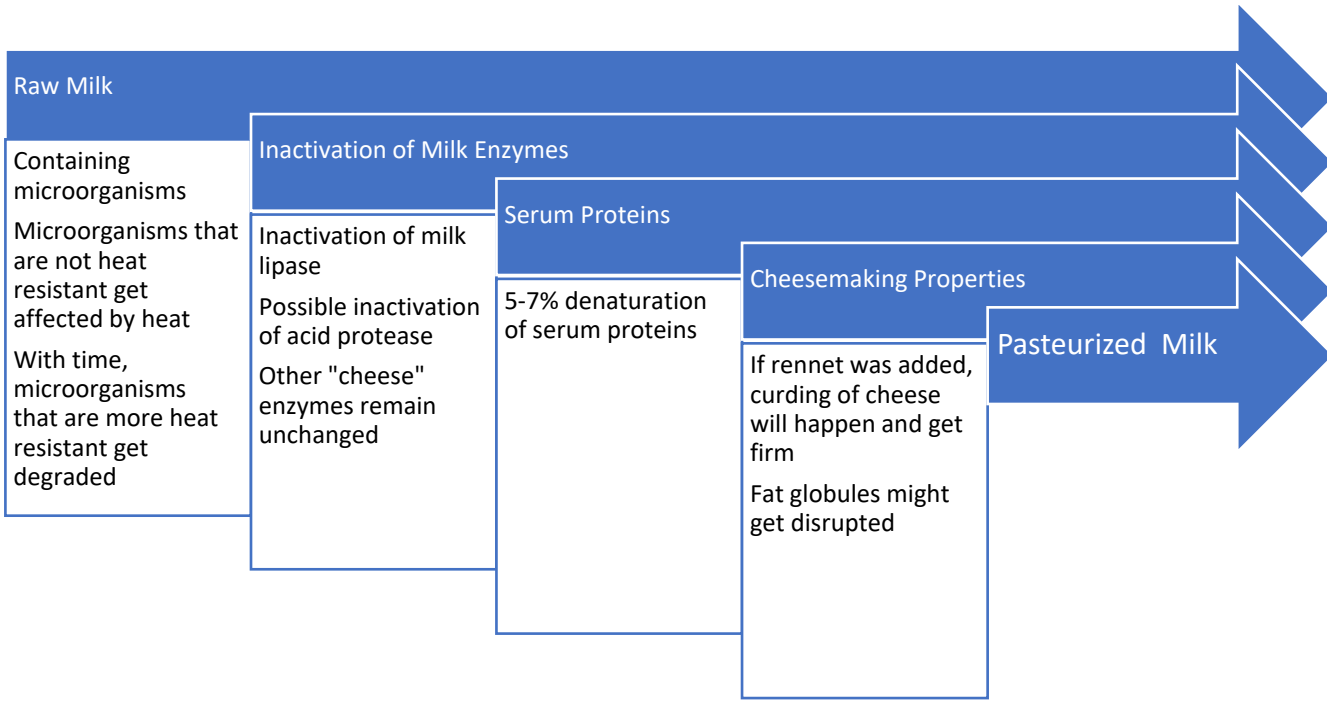

FIGURE 2: SUMMARY OF MOMENTS OF PASTEURIZATION ON THE PHYSICAL, ENZYMATIC AND MICROBIOLOGICAL CHANGES OF MIK PASTEURIZATION ADOPTED FROM (GRAPPIN & BEUVIER, 1997)

A study performed by Roca *et al.* (2010) aimed at investigating the thermo- stability of quinolones in milk. It was done through applying different heating temperatures and different heating durations (72ºC for 15 s; 120ºC for 20 min; and 140ºC for 4 seconds) on milk inoculated with five antibiotics belonging to the quinolone family: enrofloxacin, ciprofloxacin, norfloxacin, flumequine and oxalic acid. Upon extraction of the quinolone residues and testing through HPLC, results have shown that quinolones are heat stable and resistant to many different heat treatment conditions, and that enrofloxacin molecules get more inactivated at higher temperature (120ºC and 140ºC) compared to lower

temperatures (72ºC). In another study where identical quinolone-containing milk samples got subjected to different pasteurization techniques, it was seen that the maximum losses were seen with pasteurization at 120ºC for 20 mins, with maximum losses of concentration of 12.01% for norfloxacin and 12.71% for ciprofloxacin. (Roca, Castillo, Marti, Althaus, & Molina, 2010).

Quinolones residues are stable when subjected to different heat treatments when milk containing quinolones is stored at 20ºC after being subjected to heat treatment. Quinolones get degraded by <10% only (Różańska & Osek, 2013). This high stability of quinolones in milk and dairy form a noteworthy risk on the human health since the residues of these antibiotics may stay in milk after dairy processing, therefore, they can reach consumers. Another recent research study aimed at studying the transfer of several antibiotics including ENF from goat's milk into fresh cheeses and their probable effect on the food safety of consumers found that enrofloxacin is heat stable and slightly affected by the process of pasteurization, whereby the percentage reduction range of goat milk upon pasteurization is 3.4±5.8% (Quintanilla, P., Domenech, Escriche, Beltran, & Molina, 2019) .

1.6.2 Effect of Skimming and Milk Matrix:

Milk skimming is the process of removing fat from the milk, and it is done by boiling the milk in adequate stainless-steel food-grade containers to start the separation of the milk into two layers with the upper layer having most fat globules (cream), and a bottom layer with smaller and minimal fat globules making it skimmed milk. The upper layer will be removed, whereas the remaining bottom layer will be the skimmed milk that will be consumed either as raw skimmed milk or will be processed into a variety of fat-free or low-fat dairy products (Shelver, Lupton, Shappell, Smith, & Hakk, 2018).

To determine the effect of skimming of milk on fluoroquinolone residues, a study was performed where milk was inoculated with different antibiotics. Then the samples were extracted using the solid-phase extraction method and the residue level of antibiotics was determined using the liquid chromatography coupled to tandem mass spectrometry (LC-MS). This study showed that the raw milk samples that contained enrofloxacin have undergone changes that led to the total degradation of the enrofloxacin upon skimming

(Kantiani, Farré, & Barceló, 2011). Another study showed that upon skimming of the whole milk, the concentration of ciprofloxacin was lower in the milk fat compared to the whole milk initial concentration. And upon comparing the ciprofloxacin distribution between the skimmed milk, whey and curd, it was shown that ciprofloxacin exhibited dose dependency with over 100-fold concentration range. Ciprofloxacin concentration in the curd decreased only by 3%, and a high concentration of ciprofloxacin was found in the curd when moisture content was 70%. All of the above-mentioned indicate that if skimmed milk was contaminated with ciprofloxacin, it would be mostly concentrated in the curd. (Shappell et al., 2017a) .

1.6.3 EFFECT OF CURDING:

Whey is a by-product of the cheese-making process. It is used in the manufacturing of a variety of foodstuffs intended to be consumed by humans, particularly in the manufacturing of supplements and specific products targeted for athletes, animal feeding, as well as agricultural applications (Carvalho, Prazeres, & Rivas, 2013). Antibiotics could be reserved in different proportions in the milk whey or curd, based on the ability of these chemicals to interact with the fat and/or protein fraction of the matrix, also according to their physicochemical properties. (Sniegocki, Gbylik-Sikorska, & Posyniak, 2015).

A study inoculated the milk with 18 veterinary antibiotics to assess how they got affected by the cheesemaking steps, curding specifically. The aminoglycoside, quinolone, and tetracycline families showed the highest relative reduction in the activity of the antimicrobial. Whereby, for enrofloxacin and ciprofloxacin- a metabolite of enrofloxacin- the antimicrobial activity variation in whey from spiked milk (WSM) with respect to spiked whey samples (WNM) was 100% and ranging from 84 to 100% for tetracyclines and aminoglycosides signifying that antimicrobial activity of enrofloxacin decreases drastically due to cheese making (Giraldo, Althaus, Beltrán, & Molina, 2017).

In another study, goat milk was intentionally inoculated with a variety of antibiotics including enrofloxacin, then pasteurized and processed to cheese. It was evident that the retention of the antibiotic was 51.1 ± 8.8%, which indicates that the

transfer rate of these antibiotics from milk to cheese is relatively high, with a low safety margin , especially when consumed over a lifetime (Quintanilla et al., 2019).

1.6.1 EFFECT OF STORAGE AND PRESERVATION:

The stability of the quinolones residues upon storage has been studied by testing for six quinolones - enrofloxacin (ENR), ciprofloxacin (CIP), difloxacin (DIF), danofloxacin (DAN), sarafloxacin (SAR), and flumequine (FLU) and tested using UPLC-MS/MS in raw milk stored under a variety of conditions to inspect the degradation of quinolones upon milk storage. Storage conditions encompassed variety of different combinations of temperatures and durations (-80°C for 1, 7 and 30 days; -20°C for 1, 7 and 30 days; 4°C for 4, 8, 24 and 48 h), defrosting temperatures at 25, 40 and 60°C after storage at –20°C for 24 h; freeze–thawing for 1, 3 and 5 times after storage at –20°C with a defrosting temperature of 40°C. Results have shown that upon storing at -20ºC for 30 days, enrofloxacin remained stable in milk unlike other quinolones. Also, enrofloxacin has shown minimal degradation upon storage at –80°C for 30 days. When studying the effect of thawing on the stability of enrofloxacin residues in milk, results have shown that the antibiotic enrofloxacin is stable in different thawing conditions (25, 40 and 60ºC for 10 minutes after being stored at -20ºC for 24 hours. Thus, this study concluded that any thawing temperature below 60°C does not impact the stability of quinolones in milk (Chen, Wen, Wang, Zheng, & Wang, 2016).

Also, in the same study, the effect of the frequency of cycles of freezing and thawing on the stability of quinolones in raw milk was studied. For that purpose, milk samples were frozen at –20 and later thawed at 40°C for 1, 3 and 5 cycles after being frozen at –20°C. Results have shown that all the quinolones tested, including enrofloxacin, remained stable after five freeze–thaw cycles. Only sarafloxacin has shown some degradation after repeating the freeze-thaw cycle for five times. However, due to the lack of enough evidence and studies in the literature studying the effect of the cycles of freezing and thawing on the stability of quinolones in milk and dairy, it was advised that for precise detection of quinolones in milk, it is best not to freeze and thaw the samples more than three times (Chen et al., 2016)

Due to many practical, technical, and economical situations, cooling is not available for proper preservation of the milk; thus, preservation methods other than cooling are important to be studied since these may decrease the quality of the milk (Upadhyay, Goyal, Kumar, Ghai, & Singh, 2014). Raw milk was inoculated with different preservatives (potassium dichromate, sodium azide, sodium thiocyanate , bronopol, methanol) and stored at room temperature for 24 h. Results have shown that ciprofloxacin was unaffected by the presence of the preservatives. However, the other quinolones got degraded by their presence with varying degree of degradation. Particularly, the percentage recovery of enrofloxacin was 100% when sodium thiocyanate was used for preservation, 105% when sodium azide was used, 85% when potassium dichromate was used, 95% when bronopol was used, and 93% when methanol was used. Thus, it was evident that potassium dichromate exhibits the strongest effect on enrofloxacin in milk during non-cooling storage techniques, and that it is stable when the other four previously mentioned preservation chemicals were applied (Chen et al., 2016).

1.7 GAPS IN THE LITERATURE:

Maximum residue limits (MRL) for pharmacologically active components in food of animal origin, including milk, are listed by the Annex to Commission Regulation (EU) No. 37/2010 and as well as the joint FAO/WHO Expert Committee on Food Additives and Contaminants (JECFA). However, to date, no maximum residue limits have been set for food produced from milk. The absence of these values may complicate the policymaking by the official control entities, as well as the international trade arrangements. Literature available to date studying the transfer of drugs residues from milk to its derivatives such as yogurts and cheese, yogurts, etc., and their distribution among their resultant components such as the whey and curd, is scarce and mainly focused on antiparasitic qualities. Only few studies have focused on the relation between low concentrations of antibiotics residues in milk and their adverse technological effects on dairy processing (Cabizza et al., 2017) .

The U.S. FDA issued a risk-assessment entitled “Multicriteria-Based Ranking Model for Risk Management of Animal Drug Residues in Milk and Milk Products”, and gaps in

the available data were spotted, such as the nonexistence of information about the drug residue distribution in milk products. Data that describes the distribution and translocation of animal drugs in milk and its byproducts upon processing is essential to study the potential effect of human exposure (FDA, 2015).

It is difficult to study the effect of human exposures to drug residues from cow milk products because there is a very wide range of products, including various cheeses, sour cream, ice cream, yogurt, whey protein supplements, and more than 35 others, that are derived from milk or whey. It is important to understand the factors that affect the distribution and/or concentration of animal drug residues among the various milk fractions which will evident in better assessment of the risk for potential human exposure (Shappell et al., 2017b) .

Regulatory bodies have set the MRLs of antibiotic residues allowed in many of the food products. However, the MRLs only apply to the quantity of residues in the raw food commodity prior to processing and does not take into consideration the changes caused by the processing itself. Since most foods of animal source are not consumed raw, rather they undergo a variety of processing steps before consumption, it is critical to identify the effect of different treatments on the residues when determining MRLs, assessing human exposure, and evaluating toxicity (Tian, Khalil, & Bayen, 2017) .

1.8 METHODS FOR THE QUANTIFICATION OF ANTIBIOTIC RESIDUES IN MILK AND DAIRY PRODUCTS

Highly advanced and sophisticated equipment are needed to be able to perform quantitative and qualitative analysis of antibiotic residues in dairy milk and its products. Several analytical methods have been established to detect these residues. Techniques for the analysis of antibiotics residues in milk could be categorized into two categories. The first category includes the Delvo test, the direct methods for analysis such as enzyme-linked immune sorbent assay (ELISA), and the multi-channel immune-sensors; whereas the second category includes methods involving extraction/ clean-up steps and depends on chromatographic techniques for collecting data (Raza & Kim, 2018).

1.8.1 SCREENING METHODS:

There are different methods that allow for the screening of antibiotic residues. The classic method used to detect the presence of specific antibiotics in a sample is the microbiological assays; however, it has shown to present limited sensitivity in relation to the concentration of antibiotic of concern, although they require the use of specific equipment. The simplest screening tool consists of the bioassay techniques which have the tendency to show cross reactivity for analogs with similar structure to that tested; thus, delivering semi-quantitative measurements of the antibiotic residues and that is due to their simplicity and lack of structural information. For that reason, it is important to compare the positive results shown through the simple screening techniques with more reliable, selective and sensitive techniques. Physical and chemical assays detect the desired antibiotic of concern through its specific characteristics such as the size of the molecule, its binding characteristics, charge or reactive properties. The antibiotic is purified from other impurities in the sample. Then, the isolated molecule is analyzed using more sensitive equipment. Chromatographic techniques have proven to be the most accurate and reliable techniques that can give solid screening results for the detection of antibiotic residues in different food matrixes including milk and dairy products. When screening for residues of antibiotics, it is important to compare the positive results that show in screening methods with the results of other reliable, selective and sensitive techniques. Chromatographic methods offer more solid based results for the screening of antibiotics in milk and dairy products (Parthasarathy, Monette, Bracero, & S Saha, 2018).

1.8.2 EXTRACTION AND CLEANUP PROCESS:

The complex milk matrix creates a challenge for the extraction of veterinary drugs in milk and dairy products, since additional steps should be included during the extraction phase and clean-up steps are essential to ensure the cleanliness and the performance of the machine used for detection. A wide variety of extraction platforms is available and could be used for the extraction of analytes such as liquid-liquid extraction (LLE), pressured liquid extraction (PLE), solid-phase extraction (SPE), solid-phase microextraction

(SPME), matrix solid-phase dispersion solid phase extraction (MMISPE), and matrix solid-phase dispersion solid phase extraction (MMISPE).

SPE can be performed via modified (QuEChERS) which stands for "quick, easy, cheap, effective, rugged, and safe" methodology. In the QuEChERS method, two steps are essential to extract the compound of interest. The first constitutes of solvent extraction, while the second is dispersive SPE (Raza & Kim, 2018). QuEChERS technique is the most recently created method for extraction. However, in some cases, a high level of dilution might be needed (roughly 50-fold dilution), which reduces the reliability of the quantification process of antibiotics and other veterinary drugs (Zhang et al., 2019).

1.8.3 CHROMATOGRAPHIC METHODS

The quantification of residual antibiotics in the food matrix relies mostly on chromatographic techniques such as the Liquid Chromatography-Mass Spectrometry (LC-MS), High Performance Liquid Spectrometry (HPLC), turbulent flow chromatography coupled with tandem mass spectrometry (TFC-LC-MS/MS), and UHPLC-time-of-flight (TOF) MS. For the quantification of antibiotic residues in dairy samples, HPLC has been broadly used and has shown to be of the best analytical tools with respect to efficiency, detectability, sensitivity, and performance. Combining HPLC with mass spectrometry has shown increased detectability, precision and accuracy of antibiotic residues in milk and dairy. Also, it has shown to be efficient in the detection of both multiclass and single residues of antibiotics. The gold standard for the detection of antibiotic residues in milk and milk derived products and dairy is the Mass Spectrometry (MS) or MS/MS (Raza & Kim, 2018).

1.9 AIM & OBJECTIVE OF THE STUDY:

Our study aimed at studying the effect of various cheesemaking steps on Enrofloxacin residues in bovine milk and the resulting most commonly consumed Middle Eastern cheeses. The goal of our study is to assist the local authorities in the Middle East region to establish antibiotic residue limits for raw milk according to the dairy product that it will be processed to. Also, our results will help the dairy industries to decide on which product to process the raw milk to whenever they receive a contaminated raw milk

batch with antibiotics. As such, their end dairy products will be conforming to the regulations.

Chapter Two

METHODS AND MATERIALS

2.1 PRE-EXPERIMENTAL PHASE:

An extensive research was conducted prior to the initialization of the study, and based on that, the matrix (milk and dairy), antibiotic (enrofloxacin) and the laboratory (Lebanese Agriculture Research Institute) were chosen. Below are all the details.

2.1.1 CHOICE OF THE MILK SOURCE

About two-thirds of the milk and dairy choices in Lebanon and neighboring Arab countries is from cattle, specifically bovine, and the remaining one-third is from buffaloes, goats, camels, and sheep (Wilson, 2017). In addition, bovine milk is available all year long (Alqaisi, Ndambi, Uddin, & Hemme, 2010) .

2.1.2 CHOICE OF ANTIBIOTICS

A phone-based interview was carried out by a colleague from the Lebanese University among vets in Lebanon using a database obtained by the Ministry of Agriculture to inquire about the antibiotics that they frequently use. It was found that the most commonly used antibiotics for cows are gentamicin, enrofloxacin, tylosin and tetracycline.

2.1.3 CHOICE OF THE LAB:

The Lebanese Agriculture Research Institute (LARI) - 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. (*Lari.*) .

2.2 EXPERIMENTAL PHASE:

2.2.2 CHEESE MAKING PROCESS:

The whole cheese making process from the inoculation of the raw milk to reach the final products was performed at the Research & Development (R&D) Department of Dairy Khoury, a major dairy industry in Lebanon. Equipment available and used at the "pilot lab" of the industry are of the highest technological advances and were practical and flexible to use. This made us imitate the standard procedures done at the industrial level in Lebanon.

2.2.2.1 MILK INOCULATION:

450 kgs of antibiotic-free bovine milk was collected from an untreated, healthy flock of 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 225ml enrofloxacin (Enrojat, Montajat, KSA) were added to the initially antibiotic-free milk sample, whereby the concentration of enrofloxacin in the inoculated milk was 4800ppb . 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 *Figure 1*.

2.2.1.2 CHEESE MANUFACTURING:

After the intentional inoculation of the milk with enrofloxacin , the milk got parted into three batches, whereby each batch has undergone different processing conditions to assess the effect of different processes on the antibiotic enrofloxacin. In the first batch (A), 100 kgs of the spiked milk has undergone holder (vat) pasteurization (63ºC for 30 minutes) as shown in *Table 2*.

In the second batch (B), 50kg of the inoculated milk was heated at 55ºC 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 quality dairy products. After that, the resultant milk mixture has undergone pasteurization using the

holder method (63ºC for 30 min). In the third batch (C), 250 kg of inoculated full fat milk underwent High Temperature Short Time (HTST) pasteurization (72ºC 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 40ºC), 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 to drain considerable amount of water from the cheese 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 70ºC (for a duration of approximately 35min). The resulting cheese was the Halloum, which was then salted in brining solution (12%) to yield 6% salt Halloum.

To yields the double crème cheese, the whey that was produced in the first step of the cheese production was heated to 88ºC, 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 creme cheese. The detailed cheese manufacturing process and collection of samples is presented in *Table 2*.

TABLE 2: DETAILED CHEEESEMAKING AND SAMPLE COLLECTION PROCESS

Batch A	Batch B	Batch C
Refrigerated fresh milk in the RAW-VAT-PB2		
RAW MILK Sample		
Transfer the milk to 500-VAT-PB2		
Intentionally Inoculate the milk with ENF		
Mix well for 5min		
CONTAMINATED MILK Sample		
	Transfer 3 Buckets to Separator - PC3	
	Heat to 55°C	
	Separate the Fat	
	SKIMMED RAW Sample	
100kg to Fusore 1	50kg Skimmed Milk + 50kg Full Fat Milk to Fusore 2	250kg Full Fat Milk to HTST

Heat for 63°C for 30min		
Full Fat Milk VAT Sample	**Mixed Milk VAT Sample**	**Full Fat HTST Sample**
Decrease temperature to 40°C		Transfer to 300-VAT-CURD
Add Rennet, Mix, and Wait for 25 min		
Cut the curd		
Separate the Whey		
Collect Whey Sample (A)	**Collect Whey Sample (B)**	**Collect Whey Sample (C)**
Baladi Production - Draining in Cups		
Collect Baladi Cheese (A)	**Collect Baladi Cheese (B)**	**Collect Baladi Cheese (C)**
Akkawi Production - Pressing in Cloth (30 Samples)		
Collect Akkawi Cheese (A)	**Collect Akkawi Cheese (B)**	**Collect Akkawi Cheese (C)**
Halloum Production - Boiling in water/serum		
Collect Halloum Cheese 0% (A)	**Collect Halloum Cheese 0% (B)**	**Collect Akkawi Cheese 0% (C)**
Brining (12%)		
Collect Halloum Cheese 6% (A)	**Collect Halloum Cheese 6% (B)**	**Collect Halloum Cheese 6% (C)**
Transfer the Whey to Fusore 1		
Heat to reach 88°C		
Add Salt and Citric Acid		
Clotting		
Double Cream Production - Pressing in Cloth		
Collect Double Crème Sample (A)	**Collect Double Crème Sample (B)**	**Collect Double Crème Sample (C)**

2.2.1.3 SAMPLING OF THE CHEESE:

Following each of the cheese manufacturing steps and the consequent production of various cheeses, samples were collected for further extraction and quantification of the antibiotic residues it constitutes. Samples were collected after each step, stored at -20°C in the dark, then got transferred to the Lebanese Agriculture Research Institute in refrigerated trucks, while keeping the same storing conditions. During extraction, 2 samples of each product (total of 64 samples) were used.

2.2.3 MILK AND CHEESE COMPOSITION:

Determination of the composition and the proximate analysis of milk and cheese was performed at the R&D Department of Dairy Khoury, and each analysis was performed in triplicates. The levels of casein, proteins and fat in milk were determined based on the method ISO 9622 of the International Organization for Standardization (ISO, 2013). Total solids were determined through gravimetric analysis after drying to constant weight at 102 ± 2ºC, based on the ISO 6731:2010 method (ISO, 2010). pH was measured using "SevenCompact, Mettler Toledo, Switzerland" pH meter ; dry matter according to ISO 5534 (ISO, 2004); protein according to ISO 8968-1 (ISO, 2014); fat (Soxhlet, 18790; pH 4.6-soluble N) sa. A summary of all the equipment and methods used to determine the proximate analysis is presented in Table 3.

TABLE 3: EQUIPMENT AND METHODS USED TO DETERMINE THE COMPOSITION OF MILK AND CHEESE

Test	Method	Equipment Used
Fat	Gerber Method	Gerber tubes & Gerber Centrifuge (Funke Gerber Nova Safety, Germany)
Moisture	Gravimetric method	Moisture analyzer (Sartorius MA35, Germany)
Salt	Titrimetric method	Mohr
	Conductivity	Saltmeter (ATAGO, Japan)

2.3 POST EXPERIMENTAL PHASE- LAB WORK

2.3.1 EXTRACTION METHOD OF ENROFLOXACIN FROM MILK AND CHESSES:

2.3.1.1 CHEMICALS AND REAGENTS:

The standard enrofloxacin, water HPLC grade, Formic acid HPLC grade, methanol and acetonitrile (ACN) were bought from Sigma-Aldrich, as well as the extraction salts used QuEChERS Salt pre-packed envelopes containing (Magnesuim sulfate anhydrous, ReagentPlus Ò, ≥ 99.5%; Soduim chloride Puriss, p.a., ACS Reagent, Reag, ISO, Rea. Ph.Eur., ≥ 99.5%; Sodium citrate dibasic sesquihydrate, squihydrate purum p.a., ≥ 99.0% (T); Soduim citrate tribasic dihydrate purriss. P.a., ACS reagent, ≥ 99.0%). Optima Ammonium Acetate LCMS was bought from Fisher Scientific. Polish tubes and dEMP tubes were bought from Agilent.

2.3.1.2 VALIDATION OF THE METHOD:

The method was validated to confirm that it can detect the enrofloxacin residues in milk and cheese after extraction. It was done through spiking samples of milk (blank-antibiotic free) with enrofloxacin at two different levels (25 ppb and 100 ppb) with enrofloxacin, each was done three times for reputability. Recovery for enrofloxacin was 93% which is acceptable knowing that the good range for recovery, ranges between 80-110% (European Union, 2002) . After trying many extraction methods, the dEMR method for extraction of the samples was chosen because it has shown to be more effective in determining the enrofloxacin residues in milk and cheeses and was cleaner for the chromatographic column and LCMS machine.

2.3.1.3 LINEARITY:

The calibration curve of the machine (Figures 3 &4) shows that the machine was calibrated.

Limit of detection (LOD): 50 ppb

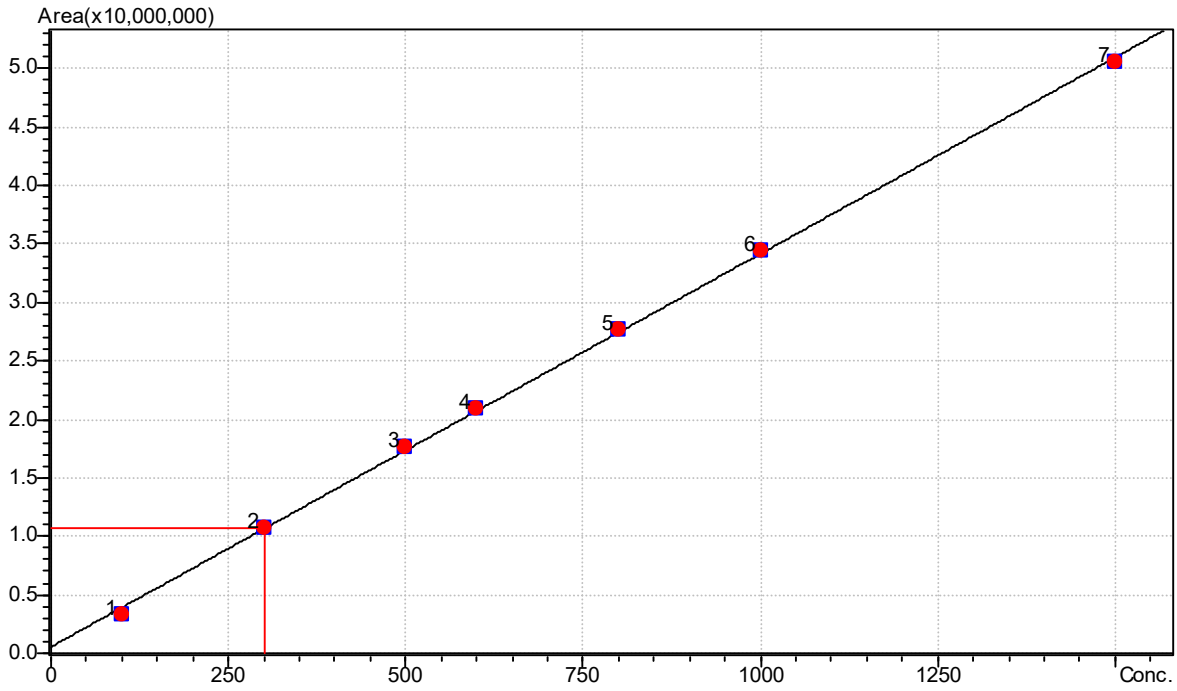

FIGURE 3: CALIBRATION CURVE OF ENROFLOXACIN (R2:0.999)

Linearity was determined through the calibration curve on the LCMS machine at 7 points (100, 300, 500, 600, 800, 1000, 1500 ppb). Parameter of the calibration curve showed good linearity, whereby the correlation coefficient R2 =0.999>0.995, which is the internationally accepted correlation coefficient on the LCMS machine (Chen et al, 2015).

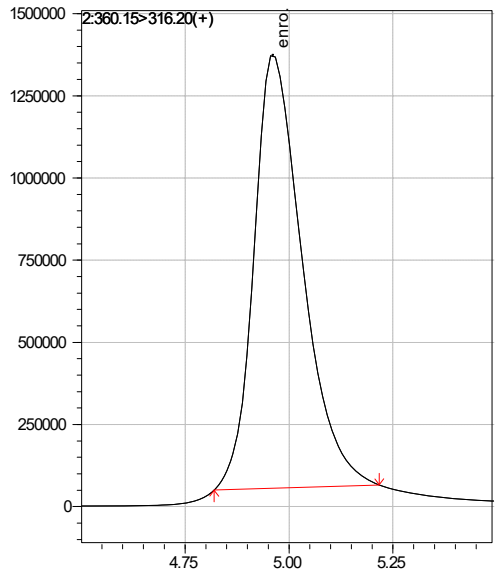

FIGURE 4: A PEAK OF ENROFLOXACIN TRANSITIIONS AS SEEN ON THE LC-MS

2.3.1.4 EXTRACTION METHOD OF ENROFLOXACIN FROM MILK AND CHESSES-DEMR (DISPERSIVE EMR) MODIFIED QUECHERS

A study published by Schweiger et al. (2018) aimed at developing and validating methods for the extraction of antibiotic residues in dairy products and evaluating the best methods to be used for this purpose (Schweiger et al., 2018). The modified QuEChERS has shown that it is very efficient for detecting enrofloxacin residues in the milk and cheese. It has been used in many studies, the most recent of which is a study published by Grabsk et al. (2019), and aimed at determining several antibiotics residues, including enrofloxacin in milk and dairy, through the modified QuEChERS method, Full Factorial Design and Liquid Chromatography-Tandem Mass Spectrometry (Grabsk et al., 2019).

A sample of the products prepared earlier got extracted using the modified QuEChERS technique. The only difference between the liquid samples (milk, whey, curd and serum) and the solid cheeses is that when extracting liquids (milk, whey, serum),10 ml of that liquid was removed from the initial sample of the liquid container after shaking thoroughly to perform the extraction on a representative sample, whereas, when the

sample was solids, 5g of the cheese was taken after well grinding the cheese block, and 5 ml of water was added to the polypropylene tube. 10 ml acetonitrile (ACN) was added and then the tube was shacked vigorously by hand for 1 min. After that, the extraction salts, QuEChERS salt, were added to the tube then shacked again by hand for 10 minutes, then centrifuged for 5 min at 5000 rpm at 4ºC. After the centrifuge was done, 5 ml of the upper layer of the polypropylene centrifuged tube were removed using a pipette and added to a dispersive EMR tube previously activated with 5 ml water, after which the latter tube undergone shaking by hand for 3 mins, followed by centrifugation for 5 min at 5000 rpm at 4ºC. Once the centrifugation was performed, 5 ml were removed from the upper layer of the polypropylene centrifuged tube and added to the polish tube, and got mixed by shaking for 1min, and then put in the polypropylene centrifuge again for 5 min at 14000 rpm at 4ºC. Lastly, the resultant liquid was filtered with pvdf of pore size 0.22 micron, to remove any residual lumps. This step is considered as a cleaning step for the extraction (Grabsk et al., 2019). After extracting the enrofloxacin residues from the cheese or milk samples, the vial containing the residues was placed in the Liquid Chromatography-Mass Spectrometry LC-MS (SCHIMADZU LCMS-8045)

2.3.2 Preparation and Storage of the Solution:

2.3.2.1 Stock Solution Preparation:

The first solution was prepared in 1000 ppm concentration and then diluted to 10 ppm (standard solution diluted) both -the standard solution and the diluted standard solutions -were stored in the freezer at -20ºC in the dark. The standard diluted solution was valid for storage for a period of 3-6 months in the same conditions, whereas, the stock solution is valid for one year after storage in the freezer at -20ºC in the dark. From the standard solution diluted, the working solution was prepared, which has a concentration of 1ppm, through dilution, working solution is for daily use (10 ppm undergone dilution to become working dilution). Enrofloxacin (1000 ppm) was prepared in methanol and 1ml NaOH (1 N). There are 7 levels to prepare the mobile phase, from 100ppb to reach the 1.5ppm (mobile phase). For the LCMS to function, mobile phases are needed: Mobile phases: A and B

Mobile phase (A): water + ammonium acetate (5mM) + 0.1% formic acid

Mobile Phase (B): methanol + ammonium acetate

Gradient:

TABLE 4: GRADIENT OF THE MOBILE PHASES AS EVIDENT ON THE GRADIENT ON THE THE LC-MS

Time (min)	%mobile phase A	% mobile phase B
0	90	10
2	60	40
7	10	90
9	90	10
10	90	10
16	90	10

All of the above-mentioned were chosen based on a study performed by Grabsk et al. (2019), because it is very similar to our study.

2.3.3 Equipment Used for Reading the Results:

2.3.3.1 Name and Type of the Machine:

The apparatus that was used for the detection of enrofloxacin residues was the liquid chromatography-mass spectrometry (SCHIMADZU LCMS-8045) with HPLC. It is a triple quadrupole LCMS/MS. The liquid Chromatography being: Nexera X2 LC-30AD Liquid Chromatography, with Degassing Unit: DGU-20A5R, Communication Bus Mobile CBM-20A and Prominence Column Oven CTO-20AC (SCHIMADZU (DGU-20A5R)).

Some specific criteria through which the apparatus functions are injection volume of 2 µl with a flow of 0.3 ml/min. The desired temperature of the column was 40ºC; whereas, the interface temperature of the LCMS was 300ºC; with nebulizing gas flow 3 L/min, and drying gas flow 10L/min.

The chromatographic column used is: 2.1 *100mm, 3µm

2.3.3.2 SPECS OF THE MACHINE AND HOW DOES IT FUNCTION:

The run time of each sample is 16 min. When the vial is placed in the LCMS, the sample gets ionized with ESI (electron spray ionization) probe. Once it becomes ionized and fragmented, the resultant that we would get is a molecular ion and other ionized fragments. Electrons get lost from the ENF, hence the enrofloxacin gets ionized and we will have remaining fragments of other molecules in the sample. Then, it gets introduced to the first quadrupole where the precursor ion gets selected. Selection is based on mass: charge (ratio) (Mass/charge)

At this point, the precursor ion is introduced into a collision cell filled with Argon gas, which is an inert gas and does not interfere in the reaction. Note that Nitrogen could be used, but Argon (Ar) is responsible for the ionization. The precursor ion gets fragmented again, and the result is a spectrum of product ions. The product ions get selected in the Q3 quadrupole and detected by electron multiplier with dynode. At this point, it is possible to detect the product of interest through its transition of ions. Multiple transitions happen at the same time, for example, multiple precursor ions and multiple transition ions (*LCMS-8045 : SHIMADZU (shimadzu corporation).*)

Note that all of the detector components are under vacuum, to avoid collision with air particles (*Why does MS require high vacuum? : SHIMADZU (shimadzu corporation).*)

2.3.3.3 Characteristics of the Transitions of Antibiotics on the Machine:

We were looking for enrofloxacin with these characteristics:

TABLE 5: DESIRED CHARACTERISTICS OF THE TRANSITIONS OF ENROFLOXACIN AS SEEN ON THE LCMS

Name of the antibiotic (retention time)	m/Z	Transitions	CE (Collision Energy)
Enrofloxacin (4.960 min)	350.15	316.2	20
		345.15	28
		342.2	24

2.4 STATISTICAL ANALYSIS:

SPSS v25 was used to perform the statistical analysis. To summarize the variables of the study and to screen out of range values, descriptive analysis was performed. Mean and standard deviation were used to describe the continuous variables. Data was assessed for normality using Shapiro-wilk. And to compare the mean difference in enrofloxacin residues after the different processing steps (pasteurizing, curding, pressing, cheese boiling, cheese salting, whey acidifying and pressing), the paired t-tests was used. Two-tailed p-values are reported.

2.5 HYPOTHESES:

H1: Skimming: milk skimming is expected to cause changes in some antibiotic residues, depending on their affinity. According to literature, the antibiotic enrofloxacin is lipophilic, thus it is expected to be prevalent more in full fat milk and not skimmed, also in milk derivatives prepared from full fat milk compared to those prepared from skimmed milk (Cabizza et al., 2017) .

H2: Pasteurization: heat treatments, like pasteurization, were reported to cause destruction among antibiotics. The higher the temperature, the more likely the antibiotics will be destroyed. In our case, it is expected that Holder Pasteurization (63C for 30 min) will cause less denaturation than exposure of milk to HTST (72C for 15 secs) (Gajda, Kozak, Sikorska, & Posyniak, 2018)

H3: Curding/rennet addition: due to the fact that milk fat is concentrated in the curd, lipophilic enrofloxacin is expected to be higher in the *Akkawi* and *Halloum* compared to

double crème, which is whey based. Also, in the batch where milk was skimmed prior to cheese preparation, it is expected that less enrofloxacin will be present.

H4: Pressing: no previous data has been published regarding the mechanical alteration of antibiotic. Pressing will remove some water and fat and thus, it is expected that hydrophilic antibiotics would be slightly affected in this step.

H5: Salting: no previous data has been published with this regard. Adding salt will extract some water out of the cheese by osmosis; thus, given that the salt concentration in the brine is at 6% only, it is expected that hydrophilic antibiotics would be slightly affected in this step

H6: Acidification: the degradation levels of antibiotics are higher in neutral pH. Thus, it is expected that acidification will not affect the antibiotic residues.

Chapter Three

RESULTS

3.1 EFFECT OF ENROFLOXACIN ON CHEESE FORMATION:

The inoculation level of the antibiotic enrofloxacin (4800 ppb) that we used did not affect the cheese making process in all three batches A, B, and C.

3.2 MILK AND CHEESE COMPOSITION OF THE DAIRY PRODUCTS:

The proximate analysis that was performed at Dairy Khoury, in specific the moisture and fat composition of both the milk and cheeses produced in Batches A, B, and C, is represented in tables 6 and 7. Inoculating the full fat milk with enrofloxacin did not affect neither the fat content nor the moisture content of the milk. Skimming led to a decrease in the fat content in the full fat milk from 3.50 ± 0.03% to 0.08± 0.02% in the skimmed milk (Table 6).

TABLE 6: MEAN MOISTURE AND FAT COMPOSITION (±STANDARD DEVIATION) OF INOCULATED FULL FAT AND INOCULATED FULL FAT AND INOCULATED SKIMMED MILK.

Product	Moisture Content		Fat Content	
	Mean	SD	Mean	SD
Raw Milk- Antibiotic Free	88.06	0.18	3.47	0.03
Full Fat Inoculated Milk	87.89	0.24	3.5	0.03
Skimmed Inoculated Milk	90.84	0.04	0.08	0.02

Also, the cheese making of milk into various cheeses, did not cause a significant difference in neither the fat nor moisture content between the three different batches of milk with their specific conditions, as shown in Table 7.

TABLE 7: MEAN MOISTURE AND FAT CONTENT (±STANDARD DEVIATION) OF THE WHEY AND THE CHEESES BALADI, AKKAWI, HALLOUM (0% AND 6% SALT) AND DOUBLE CRÈME IN THE THREE BATCHES A, B, AND C

Product		Moisture Content (%)		Fat Content (% Dry Matter)	
		Mean	SD	Mean	SD
Baladi Cheese	Batch A	63.11	1.89	17.03	0.85
	Batch B	63.04	1.31	12.67	0.76
	Batch C	61.44	1.06	17.4	0.6
Akkawi	Batch A	56.92	0.26	20.03	0.45
	Batch B	61.24	0.69	13.33	0.28
	Batch C	58.72	0.25	18.9	0.17
Halloum Cheese 0%	Batch A	46.27	1.17	25.3	1.57
	Batch B	47.97	2.94	18.17	0.76
	Batch C	45.37	0.75	23.33	1.15
Halloum Cheese 6%	Batch A	46.83	1.17	24	1.32
	Batch B	48.34	1.17	17	0.86
	Batch C	46.81	1.52	22.67	1.04
Double Crème	Batch A	58.82	3.95	19.83	2.02
	Batch B	77.49	1.12	7.83	0.28
	Batch C	58.36	3.88	19.17	1.89
Whey	Batch A	93.22	0.39	1.37	0.2
	Batch B	93.87	0.58	0.97	0.11
	Batch C	83.68	0.26	7.72	0.11

3.3 Distribution of Enrofloxacin in Milk Components:

To assess the effect of various heat treatments on enrofloxacin residues in milk, inoculated milk was subjected to two different pasteurization techniques, the holder pasteurization (63°C for 30 min) and the HTST (72°C for 15 sec). The holder pasteurization resulted in a significant 52 to 99% degradation, depending on the initial

ENF concentration (p-value:0.033). Whereas, subjecting enrofloxacin inoculated milk to HTST pasteurization led to a significant degradation rate of 99% (p-value: 0.015) (Table 8).

TABLE 8: EFFECT OF DIFFERENT PROCESSING METHODS ON ENROFLOXACIN RESIDUES IN DAIRY PRODUCTS.

		Enrofloxacin Concentration (ppb)		
	Batch	Before	After	Sig
Effect of Holder Pasteurization	Bach A	4800	64.4	0.033*
	Batch B	221.9	107	
Effect of HTST Pasteurization	Batch C	4800	58.4	0.015*
Effect of Skimming	Batch B	4800	221.9	0.012*
Effect of Curding	Batch A	64.4	232.9	0.024*
	Batch B	194.9	764.9	
	Batch C	58.4	107.3	
Effect of Pressing	Batch A	232.9	173.3	0.2862
	Batch B	764.9	474.7	
	Batch C	107.3	90.2	
Effect of Cheese Boiling	Batch A	173.3	32.4	0.033*
	Batch B	474.7	67.3	
	Batch C	90.2	47.4	
Effect of Whey Pressing and Acidification	Batch A	59.6	131	0.028*
	Batch B	116.8	204.9	
Effect of Salting		0%	6%	
	Batch A	32.4	50.7	0.679
	Batch B	67.3	96	
	Batch C	47.4	35.2	

TABLE 9: EFFECT OF DIFFERENT PROCESSING METHODS ON ENROFLOXACIN RESIDUES IN DAIRY PRODUCTS.

*: Significance Level ≤ 0.05

Milk skimming caused a 95% degradation in the concentration of the enrofloxacin residues, and the effect was significant (p-value: 0.012) (Table 8).

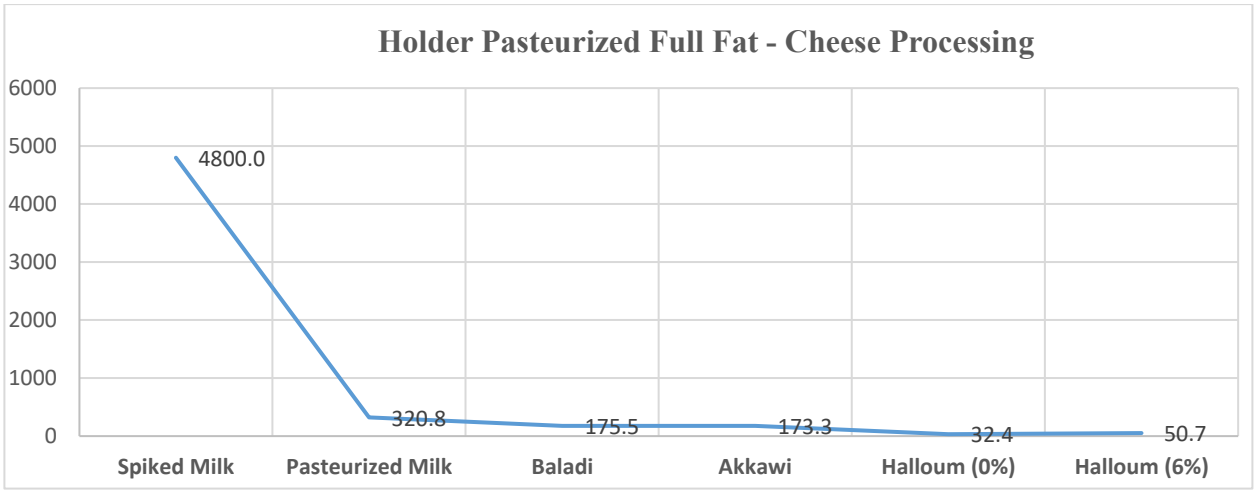

FIGURE 5: DISTRIBUTION OF ENROFLOXACIN RESIDUES IN MILK AND CHEESE OF BATCH A.

Upon adding the rennet to the milk samples to collect the curd, and thus, make the *Baladi* Cheese, there was a significant increase by 3.5 folds on average in the enrofloxacin concentration ($p = 0.024$; table 8). Pressing the *Baladi* Cheese to produce the *Akkawi* cheese did not significantly decrease (33%) the concentration of enrofloxacin ($p = 0.2862$; table 8). During this process of pressing *Baladi* Cheese to *Akkawi*, a 2-6% decrease in the moisture content of cheese was evidenced, which was not significant.

Upon boiling the *Akkawi* Cheese and collecting the *Halloum* Cheese, a significant decrease (80%) in the concentration of enrofloxacin residues was evident ($p = 0.033$), as shown in Table 8. On the other hand, salting the *Halloum* Cheese with 6% salt did not have a significant effect on the concentration of enrofloxacin ($p = 0.679$; table 8). This was accompanied by a slight change to the moisture content between *Halloum* 0% and 6%.

Boiling and acidifying the whey and collecting the *Double Crème* Cheese led to a significant increase (1.9 folds) in the concentration of enrofloxacin (p-value: 0.028, table 8, Fig. 9).

This study tracked the dissemination of enrofloxacin from inoculated milk into various Middle Eastern Cheeses. Enrofloxacin molecules were not homogeneously distributed among the various fractions of the milk derivatives (*Baladi, Akkawi, Halloum*, Whey and *Double Crème*).

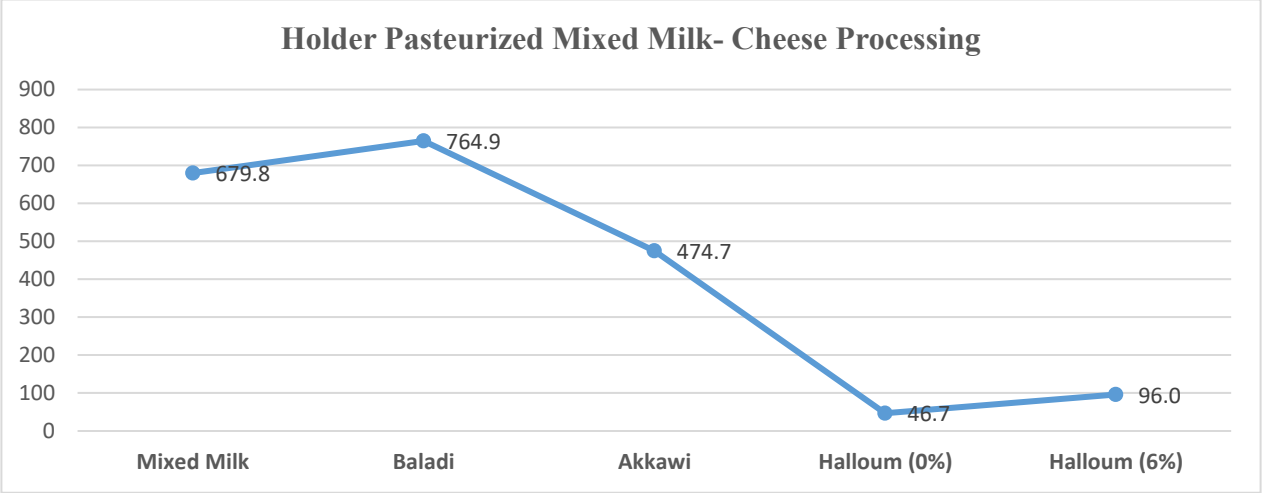

FIGURE 6: DISTRIBUTION OF ENROFLOXACIN RESIDUES IN MILK AND CHEESE OF BATCH B.

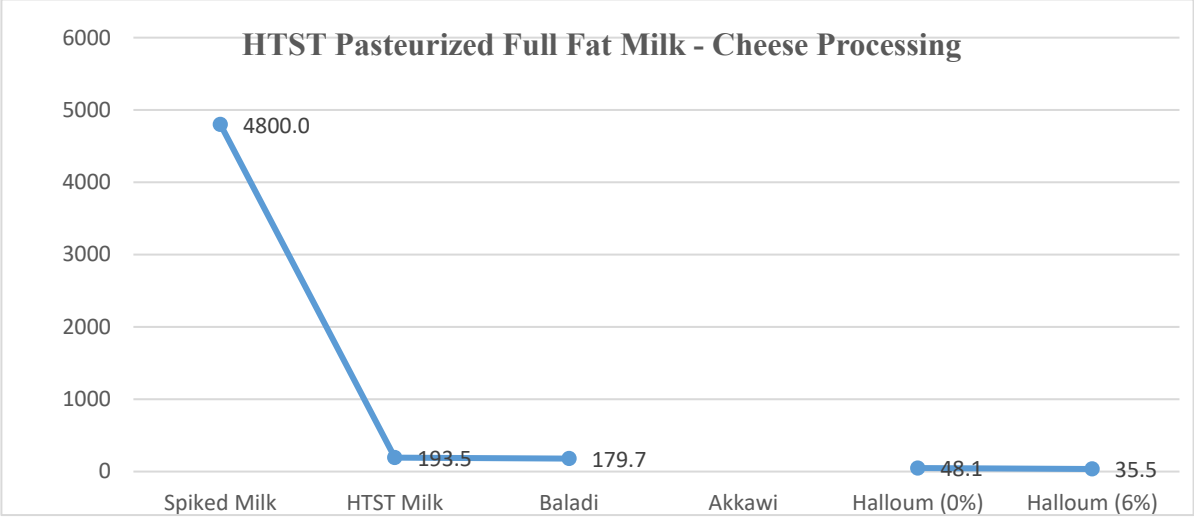

FIGURE 7: DISTRIBUTION OF ENROFLOXACIN RESIDUES IN MILK AND CHEESE OF BATCH C.

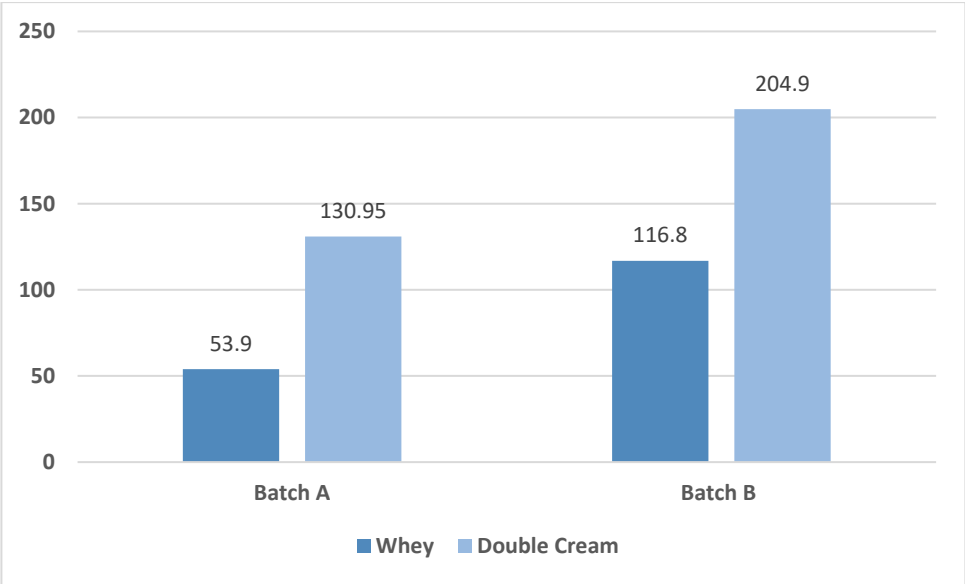

FIGURE 8: DISTRIBUTION OF ENROFLOXACIN RESIDUES IN WHEY AND DOUBLE CRÈME.

The concentration of enrofloxacin residues doubles when the whey is processed through acidification and pressing into *Double Crème* Cheese, as shown in Figure 8.

Chapter Four

DISCUSSION

4.1 ENROFLOXACIN DISTRIBUTION IN DAIRY PRODUCTS:

Studying the thermal stability of drug residues particularly enrofloxacin residues in milk and its derivatives is of critical importance for the public health due to its potential health hazards. Using different thermal treatments to heat inoculated milk with enrofloxacin causes different degradation levels. According to a study performed by Roca *et. al.* (2010), which aimed at investigating the thermo-stability of quinolones in milk, by applying different heating temperatures and different heating durations (72^{o}C for 15 s; 120^{o}C for 20 min; and 140^{o}C for 4 seconds), quinolones, particularly enrofloxacin, was reported to sensitive to destruction when subjected to higher heat temperature and not to longer time durations. Results of our study are in line with the results of Roca et al. (2010) with this regard. However, in the aforementioned study, enrofloxacin has shown great thermo-stability, whereby the degradation rate of enrofloxacin in milk was 12%, compared to 121.6% in our study. This could be attributed to the fact that the milk sample used in Roca et al. (2010) study was inoculated with a combination of quinolones (enrofloxacin, ciprofloxacin, norfloxacin, flumequine and oxalic acid) unlike our study, also due to possible discrepancies in the lab extraction methods used, since they used the HPLC to read the results, whereas, we used the LCMS which is the gold standard for the quantification of antibiotic residues in food. Another study performed by Quintanilla *et al.* (2019) studied the effect of pasteurization on enrofloxacin in goat milk showed that enrofloxacin is heat stable and is minimally degraded upon pasteurization. This result does not confirm with the result of our study; however, this inconsistency could be because the milk used in our study is bovine milk, however that in the lastly mentioned study is goat milk. Also, they inoculated their goat milk sample with MRL level of antibiotic, unlike our study where we used higher inoculation levels. Nonetheless, they used a stomacher and calf rennet to prepare the cheese, which is unique to their own study.

When observing the effect of milk skimming and fat removal on the concentration of enrofloxacin, our results showed that skimming significantly decreased ($p = 0.012$) the concentration of enrofloxacin, whereby the concentration of enrofloxacin decreased from 4800ppb in the full fat milk to 221.9ppb in the skimmed milk. This could be attributed to the fact that the enrofloxacin is lipophilic and thus was removed with the upper fatty layer of the milk. In a study performed by Lupron *et al.*, (2017), which aimed at studying the partitioning of several antibiotics and metabolites in the different fractions of milk and the possible milk products produced and suggested the degradation of enrofloxacin upon skimming.

In our study, curding caused a significant increase by 3.5 folds in the concentration of enrofloxacin ($p = 0.024$). Previous studies in the literature reported that enrofloxacin had affinity to the curd (Shappell et al., 2017; Shelver et al., 2018), which confirms our finding.

Pressing the *Baladi* Cheese to get *Akkawi* Cheese did not significantly decrease the concentration of enrofloxacin. This is due the fact that when pressing, only slight amount of water and fat will be removed (Table 7). (Giraldo, Althaus, Beltrán, & Molina, 2017c)

When boiling *Akkawi* to make *Halloum*, a significant decrease in the concentration of enrofloxacin was found. This decrease does not confirm with the thermostability of quinolones reported by Roca et al. (2010) (Quintanilla, Paloma, Doménech, Escriche, Beltrán, & Molina, 2019)(Roca *et al*., 2010). However, this discrepancy could be attributed to the fact that *Akkawi* has a different matrix that the milk used in both aforementioned studies.(Quintanilla *et al*., 2019)

Salting slightly affected the moisture and fat content of *Halloum* (Table 7). This justifies its non-significance effect on ENF.

It is worth highlighting that the MRL levels set by international agencies ensures that there are no health drawbacks of consuming milk contaminated with enrofloxacin within the acceptable levels. However, it does not take into account the change induced upon processing the milk into cheeses and does not take into account that enrofloxacin

residues in cheese are more concentrated than milk. Although this study has shown a remarkable increase in the concentration of enrofloxacin in the *Halloumi* Cheese and *Double Crème*, however, further studies are needed to study the consumption pattern of these cheeses among different age groups, and correlate it with the potential health hazards it might cause.

4.2 STRENGTHS OF THE STUDY:

Our research study is novel since it is the first in the region to assess the effect of various processing steps on enrofloxacin residues in widely consumed Middle Eastern cheeses. This will help the regulatory bodies in setting the MRLs of enrofloxacin in cheeses. Also, the cheese making procedure was performed at Dairy Khoury, an ISO22000:2005 certified facility, which reflects the cheese manufacturing at an industrial level. In addition, enrofloxacin is among the most commonly administered antibiotics to the cattle in Lebanon, and bovine milk is the main type of milk used in dairy industry. Furthermore, LCMS used in our study is considered as the gold standard for the quantification of antibiotic residues in foodstuff. Also, our analytical work was performed at LARI, the certified laboratory in the country to measure antibiotic residues in foods.

4.3 LIMITATIONS OF THE STUDY:

Results in our study included some outliers, this could be attributed to the complexity of the matrix of choice, milk and its derivatives.

This study has been performed through intentional in vivo inoculation of the milk with antibiotics, however, we did not try producing cheese from milk that it is contaminated with enrofloxacin. Although we were able to track the degradation of enrofloxacin in bovine milk and cheeses, however we did not track the degradation of the metabolites of the antibiotic, which could also possess a food safety and health risk on consumers.

Also, due to the COVID-19 pandemic, lockdown induced, current road situations in Lebanon, and the difficulty of getting supplies and maintenance of the equipment

needed, we were not able of further expanding our study to include Gentamicin, as originally planned.

4.4 Recommendations for Future Research:

Future multidisciplinary research is important to track the influence of the consumption of dairy products containing antibiotics on the human gut and microflora.

Future research should inoculate the milk with MRL level antibiotic because this is what is usually accepted for further processing at the level of the industry. Also, the whole study should be performed on other antibiotics.

4.5 FUNDING:

This research study was funded by a grant from the CNRS- National Council for Scientific Research. No conflict of interest to report.

Chapter Five

CONCLUSION

Thermal treatment of spiked milk with ENF caused a significant degradation. Curding milk and processing whey resulted in significant increase in ENF among the resulting Baladi and Double Cream cheeses. Boiling Akkawi into Halloumi caused a significant reduction in the concentration of ENF. However, pressing Baladi into Akkawi, and salting Halloumi did not significantly affect the concentration of the antibiotic. Although ENF wise, Halloum was observed to be the safest, it is advised to buy cheese from reputable sources, that follow proper food safety measures throughout the food chain.

Our novel, first-of-its kind study, proved that the most commonly consumed Middle Eastern Cheeses could be prepared even from milk that is highly contaminated with enrofloxacin above MRL levels, which possesses a huge health risk for consumers, especially that in Lebanon, not all dairy industries follow the good manufacturing practices. Thus, it is critical to assist the local authorities to establish antibiotic residue limits for both raw milk, as well as cheeses, and advise on the type of dairy product that the milk should be processed into according to the concentration of antibiotic it has upon receiving it.

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