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PREVALENCE AND IDENTIFICATION OF ANTIBIOTIC RESIDUES IN DAIRY COW'S BULK TANK MILK PRODUCED IN LEBANON

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Abstract

This research was aiming to understand the actual knowledge level of the Lebanese dairy farmers concerning the prudent use of veterinary antibiotics and to evaluate the prevalence of antibiotic residues in raw cow's milk in Lebanon. A first survey concerning antibiotics and their utilization was carried out involving 100 farmers, developing a specific questionnaire. The results have shown that most of the Lebanese dairy farmers have a very low knowledge level about antibiotics. A second study to investigate the occurrence of veterinary drug residues in dairy raw milk produced in the seven Lebanese Governorates was also carried out. During a period that extended from March 2018 until December 2018, a total of 1020 dairy raw milk samples that covers big and medium size dairy farms and dairy raw milk collection centers. Each month 102 samples were collected based on the number of medium and large farms distribution. Samples were tested using a microbiological test for inhibitors (Delvotest® T), lateral flow test for residues (Charm®test) and high-performance liquid chromatography (HPLC) test. Results show that using microbiological test, 22% (220) out of 1020 collected samples were double positive for inhibitors. HPLC-DAD test results showed that out of 220 double positive samples tested with microbiological test, 143 (65%) samples were contaminated with penicillin G (53.6%), tetracycline (23.1%) and florfenicol (22.7%) with concentrations above the European maximum residue limits, ranging between 5 µg/kg and 1565 µg/kg. Charm® TRIO and AMPH tests revealed that out of arbitrary 95 positive samples tested using HPLC, 92 samples were contaminated with penicillin G (62.1%), tetracycline (43.2%), amphenicol (40%) and sulfa drugs (32.6%). Only, 3 (3.1%) samples that were found positive using HPLC-DAD appeared to be negative using Charm® TRIO and AMPH tests.

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List of Abbreviations

ADI	Acceptable Daily Intake
AGISAR	Advisory Group on Integrated Surveillance of Antimicrobial Resistance
APVMA	Australian Pesticides and Veterinary Medicines Authority
ARA	Regional Farmers Association of Sardinia
ARPAS	Regional Agency for the Protection of the Environment of Sardinia
ASL	Azienda Sanitaria Locale
ATCC	American Type Culture Collection
AVMA	American Veterinary Medical Association
BARDA	Biomedical Advanced Research and Development Authority's
BDN	Bank Data National
BPHCT	Benzylpenicillin G residues Heated to Cooking Temperature
BPNLA	Benzylpenicilloic Acid
CA	Charm® Amphenicol
CAC	Codex Alimentarius Commission
CARB-X	Combating Antibiotic Resistant Bacteria Biopharmaceutical Accelerator
CCRVDF	Committee on Residues of Veterinary Drugs. in Foods
CONTAM	Panel on Contaminants in the Food Chain
CT	Charm® TRIO
CVM	Center for Veterinary Medicine
CVM	Center for Veterinary Medicine
CVMA	Canadian Veterinary Medical Association
CVMP	Committee for Medicinal Products for Veterinary Use
DAFM	Department of Agriculture, Food and Marine
DNA	Deoxyribonucleic Acid
EC	European Commission Council Regulation
ECDC	European Centre for Disease Prevention and Control
EEC	European Union Ecological Regulation
EFSA	European Food Safety Authority
EFTA	European Free Trade Association
ELISA	Enzyme-linked immunosorbent assay

EMEA	European Medicine Agency
EPAR	European Public Assessment Report
EU	European Union
FAO	Food and Agriculture Organization
FARAD	Food Animal Residue Avoidance Databank
FBOs	Food Buisness Operators
FD&C Act	Federal Food, Drug and Cosmetic Act
FDA	U.S. Food and Drug Administration
FSCJ	The Food Safety Commission of Japan
FVE	Federation of Veterinarians of Europe
GMP	Good Manufacturing Practice
GVP	Good Veterinary Practices
HACCP	Hazard Analysis and Critical Control Point
HPLC	High-Performance Liquid Chromatography
IARC	International Agency for Research on Cancer
ICR	Institute of Cancer Research
ID	Identity card
IDF	International Dairy Federation
IDF	International Dairy Federation
IEC	International Electrotechnical Commission
IFAD	International Fund for Agricultural Development
IFT	The Institute of Food Technologists
IMI's	the Innovative Medicines Initiative's
INEQ	Control bodies for Pecorino Romano and Pecorino Sardo PDO cheese
ISO	International Organization for Standardization
IT	Italy
IU	International Unit
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JETACAR	Joint Expert Technical Advisory Committee on Antibiotic Resistance
JPIAMR	Joint Programming Initiative on Antimicrobial Resistance
JVARM	The Japanese Veterinary Antimicrobial Resistance Monitoring System in the Field of Animal Hygiene
LAB	Lactobacillus

LMICs	low-/middle-income countries
MDGs	Millennium Development Goals
MRL	Maximum Residue Limits
MRPL	Minimum Required Performance Limit
NADA	New Animal Drug Application
NARMS	National Antimicrobial Resistance Monitoring System
NCAs	National Competent Authorities
ND4BB	New Drugs for Bad Bugs program
No	Number
NOAEL	Highest dose at which there was not an Observed toxic or Adverse Effect
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
NSF	Public Health and Safety Organization
OIE	World Organisation for Animal Health
OTC	Oxytetracycline
PDO	Protected Designation of Origin
PIFs	Pacific Islands Forum Secretariat
ppb	Part Per Billion
QCA	Quinoxaline-2-Carboxylic Acid
RMRs	Risk Management Recommendations
RNA	Ribonucleic Acid
RPA	Reference Point for Action
SCC	Somatic Cell Count
SISAR	Integrated Regional Health Information System
SSN	Servizio Sanitario Nazional
TBC	Total Bacterial Count
UCACs	Community Veterinary Officer's Offices
UDHR	Universal Declaration of Human Rights
UHT	Ultra-Heat-Treated
UN	United Nations
UNI	Ente Nazionale Italiano di Unificazione
US	United States
USD	United States dollar
USDA	US Department of Agriculture

VDACS	Virginia Department of Agriculture and Consumer Services
VICH	Veterinary International Conference on Harmonization. AcronymFinder.
VMAC	Veterinary Advisory Committee

Statement of Original Authorship

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

Signature: _____

Date: _____

Premises

During the World Food Summit Plan of Action on November 1996, Food and Agriculture Organization (FAO) declare that the Food Security exists when all the people, at any time, have access to sufficient food for their diet and their preferences to lead an active and healthy life (FAO, 1996). The United Nations (UN) office of the High Commissioner for Human rights and FAO explained that all humans, must have a safe food for consumption. By safe, they mean that humans have the right to adequate food, explaining that food for human consumption must be free from adverse substances, such as veterinary drug residues (FAO, 2011_a).

Antimicrobial drugs play a critical role in the treatment of diseases, their use is essential to protect both human and animal health. However, antimicrobials are often misused for treatment and prevention of diseases in livestock sector, aquaculture as well as crop production. These actions are often associated with the potential risk of emergence and spread of antimicrobial resistant micro-organisms (FAO, 2019_b).

The definition of veterinary drug by FAO and Codex Alimentarius Commission (CAC) is “any substance applied or administered to any food-producing animal, such as meat or milk-producing animals, poultry, fish or bees, whether used for therapeutic, prophylactic or diagnostic purposes or for modification of physiological functions or behavior” (Codex Alimentarius, 2018).

Based on the Code of practice for control of the use of veterinary drugs, antimicrobial drugs are known to be powerful tools used by confident specialist, to control infectious diseases in animals and humans. (Codex Alimentarius, 1993). As well, Codex Alimentarius has established CAC/GL 16 guidelines for the enforcement of a regulatory program for control of veterinary drug residues in foods (1993).

Nowadays, the 77th Session of Executive Committee of the Codex Alimentarius Commission was held on July 2019, in which one of their discussing points is related to antimicrobial resistance. The Code of Practice proposal to minimize and contain foodborne antimicrobial resistance includes the whole prudent use of antimicrobials using One Health' approach knowing that the human health is depending from the animal health as well as the environment. The new guidelines of controlling antibiotic residues are included, also in veterinary field and food from animal origin (Codex Alimentarius, 2005).

Worldwide, many programs to control antibiotic residues in food and bacterial resistance in humans, are applied in the European Union (EU), Sweden, Netherlands, Japan, Denmark and United States (US) (EFSA and ECDC, 2016; SWEDRES-SVARM, 2015; NethMap-MARAN, 2015; JVARM, 2013; DANMAP, 2014; FDA, 2014).

In food products there is a risk of contamination by antibiotic substances and veterinary drug residues are one of major problems (Doyle, 2006). Human management of veterinary medicines is the most likely reason for drug residues. It is born by imprudent usage such as extra-label or illegal drug applications. But, failure to respect the withdrawal period, overdose and long acting drugs, is the most evident reason for unacceptable residues in food from animal origin (Beyene and Tesega, 2014).

The Imprudent veterinary antibiotic use in animals producing food for human consumption may also cause a potential threat to human health from pathogenic-resistant organisms affecting treated animals and resulting in the food supply chain reaching consumers (Garofalo *et al*., 2007; Ramchandani *et al*., 2005). Nowadays, in the dairy farming system, antibiotics are used for therapeutic purposes. For

prophylactic purpose medically important antimicrobial agents should only be used in well-defined circumstances for the prevention/prophylaxis of a specific disease risk and follow appropriate professional oversight, dose, and duration (Codex Alimentarius, 2005). The use of antimicrobial as growth promoter to improve animal feed proficiency is not included in procedure for a prudent use, in absence of a risk analysis. They are widely used to support animal health, treat and manage infections to increase the production level (Gaurav *et al*., 2014; Vishnuraj *et al.*, 2016; Tollefson and Miller, 2000). Approximately 80% of all animals producing food are currently receiving medication for part or most of their lives (Lee *et al*., 2001).

Even when milk containing antimicrobial residues present below the maximum residue limit (MRL), the risk of consuming it is a great concern to human health.

On a therapeutic level, antibiotic treatments can have negative effect not only on humans but on the environment as well. For instance, treatment doses can affect human gut microbiota (Dethlefsen and Relman, 2011; Dethlefsen *et al.*, 2008) and promote environmental bacterial resistance (Igbinosa *et al*., 2011, Baquero *et al.*, 2008, Finley *et al*., 2013, Wellington *et al*., 2013, Novo *et al*., 2013).

When residual levels of antibiotics are ingested, they might cause allergic reaction to susceptible consumers (EMA, 2008; Sundlof, 1989; Dayan, 1993), affect negatively gut microflora (JECFA, 2017; Kuppan, *et al.*, 2017; Tancrede and Barakat, 1989), trigger cancer, mutagenicity (Booth and Mc Donald, 1988; Foster and Beecroft, 2014), genotoxicity (WHO, 2019; JECFA, 2004) and promote the development of bacterial resistance (Aarestrup, 2006).

As well human contamination by antibiotic-resistant bacteria which can be direct or indirect (Price *et al.*, 2012; Coetzee *et al.*, 2016; Liu *et al.*, 2016). Specially with workers such as veterinarians, farmers, abattoir workers, food handlers and others that

are mostly directly exposed that are at high risk of being infected with antibiotic-resistant bacteria (Marshall and Levy, 2011). With antibiotic resistance and the slow development of new antibiotics are putting in danger antibiotics, leading to face the extinction (O'Neill, 2015a; WHO, 2015a). As well commensal bacteria in livestock can be found recurrently in fresh products form animal origin, serving as a container for resistant genes that have a potential of transferring to pathogenic bacteria in humans (Mena *et al.*, 2008; Diarrassouba *et al.*, 2007). Not only human health, but dairy product fermentation is also affected by drug residues, causing a negative change in flavor production and pH concerning butter manufacturing, decrease milk curdling, cheese fermentation, affecting the safety of the process and ripening (Molina *et al.*, 2003; Payne *et al.*, 2006). Working for the same goals, and according to the legislative requirements in many countries, milk is continuously controlled for the presence of antimicrobial residues (Fejzic *et al.*, 2014).

But due to poor management practices, economic factors, and farmer's lack of awareness and education, antibiotic residues are still reported to be present in milk in levels exceeding the MRLs in different regions around the world (Al Zuheir, 2012; Al Mazeedi *et al.*, 2010; Redding *et al.*, 2014).

Dairy products daily intake in Lebanon are considered to be very close to other neighboring Mediterranean countries (LACTIMED Report, 2014). They are an essential part of the Mediterranean diet with the average intake of dairy products among adults living in different areas reported to be ranging between 243.1 g/day (Raad *et al.*, 2014) and 350.5 g/day (Farhat *et al.*, 2016). Comparing the results with the guidelines, it complies with the Mediterranean region for dairy products of 1-2 servings/day (where 1 serving is equivalent to 1 milk cup or approximately 230 g) for the intake of Lebanese population.

Based on country-specific estimates of per capita milk consumption classifications, Lebanon is categorized to be among the countries which have a high intake of milk defined as per capita milk consumption/year of >150 kg (IFCN, 2006).

Fluid milk, yogurt, and, the traditional dairy product, labneh (strained yogurt) are included in Lebanese large variety dietary intake (Raad *et al*., 2014).

Resistant strains of major pathogens against gentamicin and streptomycin in raw milk samples in Lebanon, and samples containing residues below the MRL for these antibiotics were reported (Kassaify *et al*., 2013)

Lack of accurate data on the usage of antibiotics is present in Lebanon, this will necessitate the investigation of the potential presence of other antibiotic residues in milk. This data could be useful to evaluate the risk of imprudent use at farm level that could aggravate the growing threat of antibiotic resistance, especially in pathogens that were could contaminate milk (Kassaify *et al.*, 2013) Moreover exposure assessment to antibiotic residues in dairy products is a necessary step for risk assessment for the Lebanese consumers.

Chapter 1: Antibiotic usage in the food chain

1.1 ANTIBIOTIC AVAILABILITY AND DEVELOPMENT

Human, animal and plant health sectors, are sharing the responsibility of preventing and controlling antimicrobial resistance that directly or indirectly affect food chain and environment (OIE, 2019$_a$). Antimicrobial drugs are playing a major role in humans and animals life in decreasing illness and deaths caused by infectious diseases (Tadesse *et al.*, 2012; Pogurschi *et al.*, 2015). As a result, patient's life span, threatened by bacterial infections is prolonged due to antibiotics (Piddock 2012; Rossolini *et al*., 2014).

In 1920 the average life span in the United States was only 56.4 years old. In 2015 the U.S. life span is nearly 80 years (Congressional Research Service, 2016; National Center for Health Statistics, 2016). In developing countries, where sanitation is still poor and lack of hygiene is present, antibiotic is decreasing the morbidity and mortality rates caused by food-borne and different poverty- related infections, proving that worldwide, antibiotics have had a positive beneficial effect (Rossolini *et al.*, 2014).

The newest antibiotic class until nowadays that reached the market, was discovered back in 1987. After that, a lack of innovation covered the field. These days, drug pipelines contain a few number of new antibiotics (WHO, 2017$_a$; Theuretzbache, 2011; PEW, 2015).

One of the most global crises nowadays is, antimicrobial resistance. The high rate of emergence and antimicrobial resistance spreading is causing a lot of annual

losses, with expectations of reaching 10 million deaths by 2050 with an estimated economic cost of $100 trillion (O'Neill, 2014; UN, 2015).

A prudent use of antimicrobials is a must to combat effectively resistance. The target is to block the spreading of infectious diseases, preserve existing antimicrobial therapies and foster innovation of new therapies and diagnostic tools. One of the very important factor affecting the antimicrobial resistance solution is the development of truly novel antibiotic drugs to compensate the diminishing effectiveness of the existing antibiotics. However, in line for the large variety of essential failures, drug business chart model for antibiotics is being very slow towards the growing demand novelty (Rex *et al.,* 2014; Outterson *et al*., 2015; Spellberg 2014).

At first view, in response to the growing emergency, the antibiotic development pipeline has been substantially reinvigorated. Based on the Pew Trust estimations, in March 2017, 39 antibiotics where in Phase I to III of the development pipeline (Pew, 2017). Nevertheless, based on some deep investigations, the current antibiotic pipeline is very weak to fulfill the current and projected clinical needs (The Boston Consulting Group, 2017; Pew, 2017).

Almost, 13 (33%) of 39 drugs in development will translate into a marketable product, showing a very low success rate of moving the antibiotic through the different clinical phases (Simpkin *et al.*, 2017). Besides that, not all new antibiotics have the novel mechanisms of action aiming at well-validated targets that are necessary to guarantee their effectiveness against resistant pathogens (Renwick *et al.*, 2015). As well, the pipelines may contain some combinations or redeveloped existent products. Furthermore, the highest priority antibiotic resistant pathogens are not targeted by many of those drugs. An analysis done by the Pew Trust, shows that only 12 (31%) of drugs in development would be active against *Enterococcus faecium, Staphylococcus*

aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and *Enterobacter* species and would be active against a US Center for Disease Control urgent threat pathogen (Pew, 2017).

There is a big challenge between scientific and clinical advancements in antibiotic development, particularly relative to other therapeutic fields. Some profitable therapeutic ventures are the cause of exiting the antibiotic space by many large capital companies. The void is attempted to be filled by small and medium size enterprises, but the problem is based on lack of capital and resources to undertake intensive and long term research and development (Butler *et al.*, 2013; Chopra *et al.*, 2008). Therefore, the easiest getting antibiotics, such as compound redevelopments and combinations has been struck lightly. This will leave the hard part behind for discovering truly novel antibiotics with developed mechanism of action effective against most resistant pathogens (BEAM Alliance, 2015). Moreover, bacteriophages (viruses that kill bacteria) and antimicrobial peptides are also explored in order to be used as a complement to antibiotics, but for now, they are not ready yet to assist in medical production without forgetting their limited usage (Review on AMR, 2016; Czaplewski, 2016; Fox, 2013). As well, some ideas about reviving old unused antibiotics is taking place (Theuretzbacher, *et al*., 2015). Ideas have also been put forth for public financing strategies (Ling, 2015).

Furthermore, we can't forget that novel antibiotics should recuperate their costs. It is a challenge associating those different needs into a maintainable antibiotic discovery and development procedure (Harbarth *et al*., 2015; Renwick *et al.*, 2015).

Over 50 major international and national initiatives aimed at encouraging antibiotic research and development during the past decade, such as the Joint Programming Initiative on Antimicrobial Resistance (JPIAMR), the Innovative

Medicines Initiative's (IMI's) New Drugs for Bad Bugs (ND4BB) program, Biomedical Advanced Research and Development Authority's (BARDA) Broad Spectrum Antimicrobials Program and Combating Antibiotic Resistant Bacteria Biopharmaceutical Accelerator (CARB-X) (Renwick et al., 2016). Efforts by political part for combatting antimicrobial residues is in progress. Political leaders have committed to persist on actions that encourage antibiotic research and development (Mendelson *et al*., 2016; Political Declaration of the high-level meeting of the General Assembly on antimicrobial resistance 2016). The UN declared the establishment of an Inter-Agency Coordination Group on Antibiotic Resistance to provide field guidance to ensure maintainable and successful global action that affect antimicrobial resistance with an obligation to report back on progress to the United Nations General Assembly 73rd session running from September 2018 to September 2019 (UN, 2017).

A conceptual framework for evaluating incentive program and assisting policy makers in selecting appropriate incentives was presented (Renwick *et al.*, 2015). This agenda includes the necessary market criteria to attract and support antibiotics research, development investments and public health intentions that address continuous and persistent access objectives.

1.2 SHARING ANTIBIOTICS BETWEEN HUMAN AND FOOD PRODUCING ANIMALS

In 1997, a meeting was held concerning the medical impact of antimicrobial use in food animals (WHO, 1997).

This meeting was aimed to recommend to WHO the next steps for guidelines development for control and decrease antimicrobial resistance in food animals (WHO, 1997). Bacterial resistance monitoring, antimicrobial usage data collection, prudent use guidelines directed to veterinarians, producers and allied industries, and more

stringent regulatory reviews for human microbial food safety were the main risk management recommendations for the implementation programs. These recommendations helped in restructuring of the prudent use of existing antimicrobials in animal health, which also had unpredictable impacts on decreasing the commercialization of novel antimicrobial agent for use in food animal veterinary drugs.

Meanwhile, antimicrobial resistance in human nosocomial infections, similar to vancomycin resistance enterococci, methicillin resistant *Staphylococcus aureus*, and penicillin resistant *Staphylococcus* pneumonia, was threatening human medicine and human pharmaceutical pipelines were not sufficient to control this resistance threat (Shlaes *et al.*, 2004).

In 2030, a significant increase in global food demand is expected, especially for poultry and livestock production (FAO, 2002). To cover this need with healthy food animals, novel veterinary antibiotics should be used to treat, control and prevent antimicrobial resistant food borne pathogens. WHO recognized the importance of food animal antimicrobial products and the primary role of ensuring that animals should be healthy and free of antimicrobial resistant bacteria. Not only, but national policies have been stated by WHO as well, for the prudent use of antimicrobials in animals, targeting the imperative balance of the possible benefits to livestock production against the medical risk and public health consequences deriving from their use (WHO, 1997). This is consistent with the One Health approach recognizing the relationship between animal health and humans (One Health Initiative, 2019).

With intentions of preventing imprudent veterinary drugs use, international organizations such as WHO, World Organization for Animal Health (OIE), Codex Alimentarius, the World Veterinary Association, and veterinary associations, such as

the American Veterinary Medical Association, the Federation of Veterinarians of Europe, as well as a host of animal species specialty organizations, have also implemented guidelines for use of antimicrobial products in food animals (Codex Alimentarius, 2005; FVE, 2010; WHO, 1999; AVMA, 2019).

New animal drugs, are being evaluated by human microbial food safety regulatory authorities, on the basis of quality, safety, and efficacy. The safety of the animal drug is based on the consequences of administration for the target animal as well as the consumers that will ingest potential residues. Concerning antimicrobial agents, different evaluations are mandatory, such as "salmonella shedding" studies, R-factor selection in entero-bacteriaceae, or expert reports or consultations on the potential for resistance to impact human health. As result, one of the joint FAO/WHO consultation (FAO/WHO, 2000) was aiming to urge regulatory authorities to launch a risk assessment for antimicrobial drugs. This is why on the other hand, the OIE developed guidelines on risk analysis for antimicrobials used in food animals to be used by regulatory authorities (Vose *et al.*, 2001; OIE, 2019$_b$).

In most nations, regulatory authorities continue to examine animal drugs in terms of quality, safety, and efficiency. The safety of the animal drug use does not have to be associated only with the target animal that may be affected by it but also with people consuming and ingesting potential residues. However, 1997 WHO consultation risk management recommended regulatory authorities to conduct a risk evaluation for antimicrobial products (WHO, 1997).

The initial Hazard Characterization detects the particular antimicrobial resistance in a specific food animal species and determine whether there would be a need to conduct a risk evaluation by introducing three steps: release assessment, exposure assessment and consequence assessment. The release assessment evaluates

the impact of the antimicrobial agent on the human through food originating from animals. The exposure assessment measures the frequency and duration of exposure to an agent by consuming contaminated food animal. The consequence assessment examines undesirable human food-borne diseases and treatment effects. These previous steps are integrated to produce overall risk measures to direct the proper selection of label use conditions.

Additional regulatory guidelines were developed on the basis of this outline. The VICH GL27 (adopted as CVMP 644 in Europe), the APVMA Part 10 (Australia), and the U.S. FDA CVM Guidance 152 came into effect with similar guidance implemented for Canada, Japan, and New Zealand (EMEA, 2004; APVMA, 2014; FDA, 2003$_a$).

With the introduction of the 1997 WHO consultation risk management recommendations, numerous international and national organizations have worked attentively to develop and put these guidelines into action. Several stakeholders have accepted to meet the challenges. An overview of regulatory, legislative, political, food supplier, veterinary association, consumer, and public health organization stakeholder actions and recommendations that have been applied (or are being developed) for veterinary antimicrobial use will be later described in details (Aarestrup, et al., 2008; FAO/WHO/OIE. 2008; Mathew, *et al.*, 2007; JETACAR, 1999).

Together, these novel guidance official papers, have requisite animal health companies to offer, for an innovative antimicrobial product to be used in food animals, a risk assessment of the potential human medical results attributable to the choice of antimicrobial resistant food-borne bacteria in the treated animals.

An unidentified result of these guidelines is to ensure prudent criteria for new veterinary antimicrobial agents. In addition to the present regulatory quality, safety, and efficacy requirements, other features are listed as follows: preventing cross-

resistance, nonhuman antimicrobial groups (or unique analogs) are recommended; minimizing spectrum agents; reducing co-resistance and cross-resistance selection, a bactericidal mechanism is more recommended than bacteriostatic mechanism; proper label directions to guide the product end-user to decrease (and even to prevent) the selection of food-borne bacteria resistance. Parental route of administration is recommended when possible. Oral (water and food) medications can be used for group treatment in the absence of injectable products (Thomas and Amy, 2010).

There are additional risk management strategies that may affect new veterinary antimicrobial products and are defined by animal health companies in a business case evaluation. The strategies aim to protect novel veterinary drugs and guarantee their prudent use. For instance, it is preferable to preserve new products to prevent their overuse and the loss of their effectiveness due to resistance emergence. Formularies should be developed to guide which products to use or avoid based on human importance ranking records. There is a need for antimicrobial susceptibility examination methods and clinical breakpoints to facilitate laboratory testing results to direct the selection of the proper product. Sufficient diagnostic methods are necessary to guarantee that the clinical disease is related to a pathogen treatable with an antimicrobial agent for targeted therapy (e.g., to differentiate bacterial infection from a viral infection). Recommendations providing control of antibiotic use by veterinarians should be developed (Thomas and Amy, 2010).

Moreover, in order to keep some antibiotics safe from veterinary usage, a new theory to classify antimicrobial agents of importance to human medicine a report was announced in the 1999 in the Australian Joint Expert Technical Advisory Committee on Antibiotic Resistance (JETACAR) (JETACAR, 1999). FDA Center for Drug Evaluation Research Advisory Committee have developed this important ranking

concept, and resulted in a list of standards to contribute in the classification and the human ranking now is enclosed within Appendix A of Guidance 152 (FDA, 2003_a). In addition, Canada and Japan have established as well, their own national lists of important drug for use in risk assessment (Canadian Veterinary Drug Directorate, 2005).

Based on these new guidance outlines, it is mandatory for animal health companies to present a risk assessment of the potential human medical consequences related to the insertion of new microbial product. As an example, the U.S. FDA CVM Guidance 152 determines particular conditions regarding the microbiological effects of animal drugs on bacteria of human health concern in terms of the antimicrobial agent, the prescription status, administration parameters (route, individual or group, duration), examination program "coverage" and the option for a Veterinary Advisory Committee (VMAC) meeting.

The guidance document provides guidance for therapeutic uses (including prevention, control and treatment) by various routes including water, feed, injection, and infusion (intra-mammary), and outlines appropriate conditions of use. Recently, FDA CVM has issued draft Guidance 209 which proposes that the use of antibiotics judiciously means that unnecessary or inappropriate use should be avoided (FDA, 2012_b).

Although Guidance 152 can be applicable to new antimicrobial drugs, it is also relevant for product line extensions (such as a change in animal species, dose, or duration) and retrospective products.

Newly approved products by the Guidance 152 process, consist of fluoroquinolone injection and a phenicol feed additive for swine (enrofloxacin and florfenicol respectively), and a new macrolide injection for cattle (tulathromycin). On

the other hand, a 4th generation cephalosporin cefquinome (previously approved usage in Europe) was examined in 2006 by members of VMAC as not meeting the conditions of use of Guidance 152 in the United States and as the product was not approved by CVM (CVM VMAC, 2006).

This particular example explains how regulatory action prevents the development of certain antibiotic classes. Novel antimicrobial development has been to guide animal health companies in their discovery, progression and efforts to fill the pipeline. Future therapeutic products with the following characteristics will likely obtain regulatory approval for Guidance 152. It is recommended to use nonhuman antimicrobial classes or unique analogs within human use classes to reduce cross-resistance concerns in food-borne bacteria. It is recommended to reduce genetically encoded resistance mechanisms of consequence to human pathogen resistance. It is required to provide prescription or Veterinary Feed within human use classes Directive to support veterinarian stewardship. Administration via injection for a single animal should be for less than 6 days, or for groups of animals should be orally treated under specific use conditions. The United States and the European Union re-evaluate specifically approved molecules based on the regulatory guidelines. Even though the Guidance 152 or the CVMP 644 was no applied, the United States followed a risk assessment process and the European Union a Reflection Paper to show particular concerns fluoroquinolone and cephalosporin use. In the United States, the post-approval Notice of Opportunity for Hearing procedure was applied to Baytril ® (enrofloxacin; Bayer Animal Health, Shawnee, KS) water soluble for poultry, which in part followed a risk assessment process that resulted in the FDA Commissioner's decision to withdraw the approval (FDA, 2017$_a$).

In the European Union, the Scientific Advisory Group on Antimicrobials to CVMP did not follow a risk assessment process, instead, they proceeded with a Reflection Paper to examine and reduce the conditions of use on the product label for fluoroquinolones and cephalosporins (EMEA, 2006, 2009).

On the other hand, the U.S. FDA CVM withdrew its decision after having issued a prohibition order to restrict the extra label use of cephalosporins in food animals, because it was shown in public comment that a risk assessment had not been followed (FDA, 2008, 2017_b).

Nevertheless, Australia took a precautionary approach so that no fluoroquinolones would be approved for use in food animals (Aarestrup *et al.*, 2008). These actions in several key markets discouraged the development of new antibiotic analogs, especially within the fluoroquinolone and cephalosporin classes.

The 1999 Australian Joint Expert Technical Advisory Committee on Antibiotic Resistance (JETACAR) introduced in their report a new concept to recognize antimicrobial agents significant to human medicine (JETACAR, 1999).

This important ranking was planned to assist in the Consequence section of a risk assessment and was developed during a 2003 FDA Center for Drug Evaluation Research Advisory Committee meeting. The result was establishing a list of criteria to assist in the categorization and the human importance ranking now presented within Appendix A of Guidance 152 (FDA, 2003_a).

Furthermore, Canada and Japan have also set up their own national lists of importance for use in risk assessment (Canadian Veterinary Drug Directorate, 2005; FSCJ, 2016).

In 2005, a report that specified Critically Important, Highly Important, and Important antibiotic categories was issued by a small expert consultation convened by WHO. The meeting was held as an action from the Joint FAO/WHO/OIE consultation on Non-Human Antimicrobial Usage and Antimicrobial Resistance (1st Workshop on Scientific Assessment, 2003 in Geneva, and a 2nd Workshop on Management Options, 2004 in Oslo) (FAO/OIE/WHO, 2003; FAO/OIE/WHO, 2004).

The WHO list was intended to direct risk management strategies for nonhuman use antimicrobial agents. The WHO list was revised again in 2007 and in 2009 at the WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR) meeting, each of which updated the list of antimicrobial agents in the Critically Important category (WHO, 2007; Collignon, *et al*., 2009; WHO, 2009).

In 2004, a list of Veterinary Critically Important, Highly Important, and Important Antimicrobial agents which covered multiple animal species, disease indications, routes of administration, and other unique attributes was issued by the OIE surveyed Chief Veterinary Officers around the globe in response to the Joint consultation, and was approved by OIE General Assembly in 2007. Later that year, the two lists of critically important antimicrobial agents were compared in the 3rd Joint Meeting on Critically Important Antimicrobials that was held in Rome, Italy (FAO/WHO/OIE, 2008).

Different factors can lead to the contamination of food from animal origin by veterinary antibiotic residues, which might affect consumers negatively. This is why risks assessment should be calculated focusing on human food supplies.

In general, risk assessment models in veterinary medicine emphasize animal health and treatment of diseases in animals, food scientists' studies focus on the safety of human food supplies and the presence of antibiotic-resistant bacteria on food

products, clinicians and epidemiologists investigate human outbreaks caused by resistant infections for which animals are identified as primary sources, and molecular biologists examine relationships between resistant strains and the prevalence of specific resistance genes in human and animal bacteria. It is unlikely that any single study will be able to fully and accurately quantify the relationship between antibiotic use in food animals and infections in humans. At best, only crude estimates of the etiologic fraction or "impact fraction" can be made for specific links in the ecologic chain (Greenland *et al.*, 2008).

Numerous mathematical methods are proposed to determine the overall risk caused by veterinary antibiotic usage. The method should cover direct and indirect risks of antibiotic residues on consumers without forgetting the benefits of antibiotic use in animals for food productions such as decreasing bacterial shedding and development (Landers *et al*., 2012).

As an example given, the U.S. Food and Drug Administration (FDA) oblige novel antibiotics manufacturer to perform risk assessments demonstrating the safety and the effectiveness of the new drug (FDA, 2003_b). In order to evaluate potential human health consequences, FDA use a qualitative method to classify risks as low, medium or high. Those classifications are the probabilities that:

- bacteria from animals become resistant;

- the resistant bacteria will be ingested by food consumers;

- the ingested bacteria will affect the consumer negatively.

Approval decision of the drug is based on these risks estimation in addition to information about marketing status, extra-label use and intended method of use.

The FDA might adapt a high-risk drug only if they find that human health risk can be reduced. Medium-risks drugs might be approved in a condition of labeling correct restriction.

Furthermore, FDA has established more than the direct risk assessment described before, a guidance to define antibiotic residues risks when food is contaminated (FDA, 2003_a).

This guidance requires finding antibiotic residues effect on healthy human's microbiota with the presence of resistant bacteria in consumers. Not only but this guidance explains the methodology of calculating Acceptable Daily Intake (ADI) for veterinary drugs with considerable risk to human health (Landers *et al*., 2012).

For instance, in 2000, both the United States and Europe approve tulathromycin and a new triamilide subclass of macrolides as new products for food animal use within these classes; cefquinome is only approved in Europe.

Table 1: Shows the original comparative list that has subsequently been re-examined and updated to include revisions made in June 2009 by the WHO AGISAR expert meetings (WHO, 2009; FAO/WHO/OIE. 2008; Collignon *et al.*, 2009; WHO/AGISAR, 2009)

Classes used in human medicine	Classes used in veterinary medicine
Aminoglycosides	Aminoglycosides
Cephalosporins (3rd and 4th generations)	Cephalosporins
Macrolides	Macrolides
Penicillins (natural, aminopenicillins, and antipseudomonals)	Penicillins
Quinolones	Quinolones
Tetracycline (only Tigecycline)	Tetracyclines
Ansamycins	
Carbapenems	
Glycopeptides	

Oxazolidinones	
Streptogramins	
Lipopeptides,	
Drugs used solely to treat tuberculosis or other mycobacterial diseases	
	Phenicols
	Sulfonamides

It is shown (table 1) that there is a presence of overlap, on a shared-class basis, for six (aminoglycosides, cephalosporins, macrolides, penicillins, quinolones, and tetracyclines) out of 15 "critically important" antimicrobial classes. There are a variety of subclasses for these six antimicrobial classes (e.g., within the macrolides and cephalosporins).

The ranking of antibiotics based on declared importance to human medicine results in prejudice against the food animal use of specific antimicrobial classes categorized as "critically important" or meeting "focusing criteria that considers drugs of greatest priority when a big numbers of people affected with diseases for which the drug is the sole or one of few alternative therapies" (FAO, 2007)

Political regulatory on the use of antimicrobial products in food animals has been determined. The European Commission, Council Regulation (EC) No. 2821/98, withdrew the authorization of specific antibiotics, effective June 1999 (European Union, 1998_a).

On June 19, 2003, the European Council recalled the European Commission and Council on the prudent use of antimicrobial agents in human medicine and to prevent or control antibiotic growth promoters in the European Union in 2006. This was a matter of public concern rather than science aiming at decreasing healthcare-associated

infections. The Preservation of Antibiotics for Medical Treatment Act in 2009 was introduced into the 111th Congress in the United States (H.R.-1549, 2009).

Numerous restaurant chains have taken the responsibility to take account of antibiotic use issue in food animal production. Hence, McDonald's, among other restaurant chains, food companies, and even some food animal production companies, announced an antibiotic use policy for its supply chain partners (McDonalds, 2003). These policies at the wholesale and markets require animal health companies to determine a specific segment of accepted product types in the public.

Several consumer organizations and professional societies have been long concerned about the use of antimicrobial agents in food animal production as it may affect human health, food safety and the environment. Keep Antibiotics Working, a coalition of an advocacy group in the United States spreads awareness, fights against the spread of antibiotics and partners to ensure political, regulatory and business action (KAW, 2019). It is important to distinguish unfavorable antimicrobial products and uses and the reasons for that position, as well as to detect innovative products that may address those concerns.

The European Technology Platform for Global Animal Health strategic research agenda determines the recommended research to accomplish the aim of the platform, specifically, "to facilitate and accelerate the development and distribution of the most effective tools for controlling animal diseases of major importance to Europe and the rest of the world, thereby improving human and animal health, food safety and quality, animal welfare, and market access, contributing to achieving the Millennium Development Goals" (MDGs, 2000).

Appropriate development will be made by the cooperation of multiple stakeholders, such as regulatory authorities, industry representatives, OIE, academia,

and veterinary association members. They're currently focusing on developing vaccines, pharmaceuticals, and diagnostic tests for major animal diseases. The progression of pharmaceuticals is mainly important because new pharmaceuticals for animal health are limited; therefore, it is endangering the efficient control of a number of animal diseases. This innovative venture may encourage other regions to engage in future activities and may serve as a model.

Anti-infective platforms, chemistry, or lead molecules and new screening approaches are being developed by a large group of biotech, start-up, research foundation, and even "big pharma" companies (Falconer and Brown, 2009).

A specific molecule may be predicted to be effective in both human and veterinary applications at the beginning of lead identifications. Therefore, animal health companies may have to identify those unique opportunities believed to succeed in commercialization without compromising human applications.

Consumers are demanding for an antibiotic-free food, this is why different companies such as the Public Health and Safety Organization (NSF), as well as Betagro that is world's first firm certified for antibiotic-free meat 'Betagro' are world's first firm certified for antibiotic-free meat (Betagro, 2019).

Not only but major food Companies Committed to Reducing Antibiotic such as in the United States classify food from animal origins (PEW, 2016).

These companies classify the products based on its laboratory tests results. For instance, food is classified as organic when antibiotics are not used during production stages.

When it is proved that the raising system do not accept antibiotic use it is classified as “No antibiotics ever/no antibiotics/raised without antibiotics” (PEW, 2016).

Medically not important antibiotic is classified as a no medically important antibiotics (FDA, 2003$_a$). Those classified antibiotic should be labeled indicating that antibiotic drugs important for therapeutic use in humans cannot be used.

Moreover, judicious use of antibiotic as standard that follows the prudent principles usage outlined by FDA and the American Veterinary Medical Association (FDA, 2019$_a$).

A standard that follows the judicious use principles outlined by FDA and the American Veterinary Medical Association and unnecessary or inappropriate use of antibiotics should be avoided (PEW, 2016). Some antibiotic policies and company are aware of it but different classification defer from the description of other antibiotic policies and company labels may have variations of the descriptions above that might change: Antibiotic free; routine use prohibited or no growth-promoting antibiotics (PEW, 2016).

1.3 PROPER ANTIBIOTIC USE

Animals, as all life beings, could become sick during their life. Food producing animals especially in intensive farming system, such as large and small ruminants, poultry and aquaculture, are more likely exposed to antibiotics in order to prevent infectious infections or to promote their growth (Ungemach, 2000; Teuber, 2001). An important number of antibiotics used for animal treatments, are similar or narrowly identical to the ones used to treat humans (Teuber, 2001; Saad, 2016). Different animal

species that are used for other purposes such as fur, companion animals, sports animals, are exposed as well to bacterial infections during their lives, but treated separately when clinical signs are shown (Gustafson and Bowen, 1997). For instance, when a dog or a horse or other companion animals, show some clinical symptoms due to an infection, they could be monitored easily with a possibility of quarantine when needed. Hence the risk of the infection spreading in veterinary hospitals and clinics (European Union, 2015; DSAVA, 2012). This approach is not applicable when flocks or herds of animal for food production are exposed to an infection (CVO, 2014). Antibiotics responsibility is still the best choice in veterinary medicine in order to manage and treat bacterial infections in animals. With the intention of increasing the prudent use of antibiotics in animal husbandry, guidelines were issued by the United Nation Organization of UN-Office international des Epizooties (OIE, 2013) and confirmed by the European Union (European Union, 2015). These guidelines aim to protect the effectiveness of antibiotics, stop the spreading of resistant bacteria and avoid human food from those bacteria. In addition, all the interested parties involved with animal husbandry such as, the veterinary pharmaceutical industry, practitioners, breeders and farmers, are touched by those guidelines. The competent authorities are as well exhibited by the guidelines, with the main role and responsibilities of dealing with the marketing and production of veterinary antibiotics. The regulation of antibiotics usage was born in 1990s, and an establishment for maximum residue limits (MRLs) for variety of antibiotics for different animal tissues, milk, and eggs took place (Bousova, *et al.*, 2012).

Conferring to the European (EU) and Codex Alimentarius Commission standards (CAC), the MRL in milk for tetracyclines, including OTC, is 100 μg/kg (100

ppb), whereas the MRL for β-lactams is specific to the antibiotic; for example, the MRL for penicillin is 4 μg/kg (4 ppb) (Zhang *et al*., 2014; Movassagh, 2011).

The approved guidelines for the prudent use of antibiotics in veterinary medicine recommend some measures in order to ensure the final goals such as complying with the mandatory standards recommended by the national organizations, maintain the safety and effectiveness of antibiotics, decrease resistant bacteria spreading to humans, respect the maximum residue limits (MRLs) of the used antibiotic and make sure that the products of animal origins are safe for human consumption (European Union, 2015; OIE, 2013).

For the prudent use of antibiotics, the technical committee of UN-Office international des Epizooties has recommended the following criteria; describing antibiotics as hazardous substances, it should be prudently used under the supervision of professionals and experienced skilled peoples. The good veterinary practices (GVP) is the only way of using antibiotics, without forgetting that vaccines and improving husbandry conditions are a must in diseases prevention. Antibiotic usage should be strictly aimed for approved and intended use without forgetting to adjust therapy based on isolates tests from food-producing animals during their production season. An active cooperation between all veterinary antibiotic involved parties such as, veterinary pharmaceutical industry, distributors and handlers, administrative and scientific authorities, veterinary practitioners and livestock breeders and producers, is mandatory. It is imperative to remember that prudent veterinary antibiotic use is the most important part of the good veterinary practice (GVP) (European Union, 2015; OIE, 2013).

Antibiotic sensitivity creates a significant difference when prescribing an antibiotic and it is more clear with the frequency of sensitivity testing, practitioner's

skills, background and some affecting factors. The more sensitive, easy, rapid and cheap the testing services are, the less risks of increasing bacterial resistance will occur (FDA, 2013). The codes of practices, conducts and ethics should be updated with the lunching of novel veterinary antibiotics. Forcing such codes will increase the therapeutic efficacy and decrease the risk of bacterial resistance. Not only world widely used to treat bacterial infections, antibiotics are used as well in feed to improve feed utilization and animal's growth. Concerning antibiotic feed additives, they are poorly absorbed in the gut, and the big remaining quantity is excreted in animal secretions such as urine and feces leading to environment and soil contamination (Spaepen *et al.*, 1997; Kim *et al*., 2011; Ok *et al.*, 2011). This is why, antibiotic usage as growth promoter is not recommended anymore (European Union, 2005; European Union, 2003). As well, it is imperative that veterinarians, livestock breeders and practitioners should have the needed knowledge to control infectious diseases, and veterinary antibiotics effects, other than the environmental cycle (FVE, 2018).

Dairy industry is covering a big part of our food industry. With breeding technologies, average milk production and herd size had increased significantly (FAO, 2017_b; Statista, 2019) One of the common protocols in intensive farming systems is to separate the new born calves from their mothers within a day of their birth. To control infections and feed intake, newborns are placed separately and fed with a milk replacer that contains tetracycline for 6 to 8 weeks of age in order to prevent bacterial infections. Tetracycline, penicillin and sulfonamides are administered orally or by injection to prevent and treat common pneumonia and diarrhea. Intensive dairy cows farming system is putting animals under great metabolic stress because of the high population densities. Milk production is increasing very fast, as well as diseases related to this increase, affecting negatively food quality and animal welfare (Trevisi *et al*.,

2014). Differing to beef and poultry industry, dairy industry is using antibiotics for therapeutic functions (LeBlanc *et al.*, 2006). In order to treat the common diseases of mastitis, lameness, respiratory diseases and gastrointestinal disorders, antibiotics are the best choice (Sawant *et al*., 2005; Avery *et al*., 2008).

Intra-mammary use of antibiotics was frequent in numerous countries and specially the developing ones, with poor knowledge level concerning pharmacokinetics, withdrawal period and efficacy. Antibiotics cover all dairy cow's production stages, starting from heifers, lactation and dry period.

Mastitis is covering the biggest challenge of diseases in lactating dairy cows. It is caused by infection of the mammary gland in two forms, clinical or sub-clinical and differentiated by some milk composition criteria (Barlow, 2011; Ruegg, 2013; Fejzic *et al.*, 2014). The perfect mastitis treatment method relies on a good data concerning clinical signs, sensitivity tests and milk composition (CVMA, 2008). *Staphylococcus aureus*, one of the mainly bacteria causing chronic mastitis, is rarely affected by antibiotic therapy, but some studies showed that the antibiotics of choice for such infection are cephapirin, pirlimycin and amoxicillin (Pol and Ruegg, 2007). A good part of organisms causing mastitis is classified as environmental. This is why, a bad management system can lead to increase environmental mastitis, and good husbandry practices with supportive care can decrease clinical cases and make antibiotics therapy not necessary (Roberson, 2003). High yielding dairy cows are more affected by infectious diseases, especially in when resuming lactation after calving (Trevisi *et al*., 2014). As a recommendation for a healthy dry period, reducing environmental stress, applying dry cow therapy and a good nutritional support, can decrease the risk of dry cow period problems. Concerning dry cow therapy products, they are available in the market as well as treat sealants that are a healthy option to prevent mechanically

addition intra-mammary infections (Raymond *et al*., 2006). Dry cow therapy plays a positive role in decreasing the risk of mastitis in the primary lactation period, without increasing the risk of intra-mammary mastitis at calving (Cameron *et al*., 2014). When selecting new heifers for the farm, the only 2 choices available are a heifer that was raised on milk replacer with added antibiotics such as tetracycline or neomycin in order to prevent common infections in young ages, or on whole milk (Walker *et al.*, 2012). Usually, primary diarrhea and respiratory infections require antibiotic therapy in replacement heifers. The main cause of pre-weaned calve's mortality is diarrhea, and treated with ceftiofur, but the antibiotics of choice are florfenicols or tilimicosin (Raymond *et al*., 2006). Studies have shown that worldwide, about 50% of all antibiotics made are used in animal agricultural applications (Stahl *et al*., 2012). Among the antibiotics used in dairy cattle field systems worldwide, tetracylines and β-lactam veterinary classes group are the most common used (Al Zuheir, 2012; Zhang *et al*., 2014). The most commonly worldwide used broad spectrum antibiotics for prophylactic and growth promotion purposes are tetracyclines (Aalipour *et al*., 2014).

For beef cattle's, the case is different. The problem lay on beef calves when they are shipped to mass production and maintained in large groups in high densities, especially in developing countries, leading to more morbidity particularly in newly received calves. During such production system, diarrhea and pneumonia are the main risk to the flock life, knowing that many organisms can cause bovine respiratory disease, and change during the progression of the diseases (Stanton *et al*., 2010). Because of the frequent ownership changes, during their life cycle, beef cattle cannot be under good veterinary practices. This is why the known approach is based on the idea of the animals in the group are either a subject or carrier of a diseases (Gonzalez-Martin *et al*., 2011). This hypothesis is applied in USA, with 83% of cattle feedlot

receiving antibiotics through food or water (Carson, 2010). Tylosin, tetracyclines and florfenicols, are the main antibiotics used orally. They play a prophylactic role against liver abscesses, diarrhea, respiratory and foot rot diseases without forgetting their role as growth promoters at sub-therapeutic levels (Kim *et al.*, 2012; Rama *et al.*, 2016). There is a wide relation between antibiotic use and resistance in beef cattle, leading to a conflict in the results between claves treated with streptomycin, penicillin, tetracycline and the resistant Escherichia coli found in their feces (Gibbons *et al.*, 2014). Moreover, in beef mass production, fewer antibiotics are used comparing with other species of animal for food production (FAO, 2011_b).

Poultry production is increasing rapidly to cover the consumption needs of eggs and meat (Scanes, 2007). They are a very important source of animal protein in developing countries (FAO, 2007_b). Such heavy industry requires standardization in management practices concerning drug treatment practices, particularly when controlling infections and diseases. Coccidiostat are available in broilers rations, some of them are broad spectrum antibiotics of ionophores and sulfonamides. Moreover, bambermycin, bacitracin, penicillin, chlortetracycline, and virginiamycin are used as growth promoters, feed efficiency in broilers and egg layers (Ahmed and Gareib, 2016). Therapeutic and sub-therapeutic use of antibiotics is imperative in poultry industry (Geier *et al.*, 2010). Some studies show that amoxicillin and tylosin are used for therapeutic reasons, while lincosamides are used for curative and preventive purposes (Hughes *et al.*, 2008). In developing countries, poultry producers use antibiotics as prevention and curative against diseases. The antibiotic is used without veterinary prescription, leading to an imprudent use, more specifically, disrespecting withdrawal period and adverse effects to human health and environment (Sirdar *et al.*, 2012).

In aquaculture, antibiotics are very essential. They cure treated fish indirectly, by controlling bacterial growth population in a fish and promote their immune system to eliminate them (Castro *et al.*, 2008).

Sources of stress are important before prescribing antibiotics to fish, involving water quality, temperature, fish species (De Briyne *et al*., 2013).

Since 1997, the European Union have forbidden several other antibiotic as feed additives (European Union, 1997$_{c;}$ European Union, 1998$_{a}$).

Generally, when prescribing antibiotics, precautions are essential. A qualified veterinarian should prescript antibiotics based on clinical diagnosis with sensitivity test which helps to decide the best antibiotic for the case (De Briyne *et al*., 2013).

Usually when good veterinary practices are available, metaphylaxis antibiotics should never be used (Alvarez-Fernández *et al.*, 2012). When necessary, metaphylactic treatment can be prescribed based on clinical findings concerning the chronic appearance of a disease in a flock (Trevisi *et al.*, 2014). Treating sick animals individually and quarantining them is safer (European Union, 2015). Records should be kept in order to have a history about nature of infections and antibiotic used to have a better correction plan (Gonzalez-Martin *et al*., 2011). The first choice of antibiotic used should be narrow-spectrum, unless tests results show that they could be ineffective. Broad-spectrum antibiotics should be avoided as much as possible (European Union, 2015). In case of recurrent infection cases, identifying the bacteria in cause is necessary in order to facilitate the pathogenic microorganism eradication and taking into consideration antibiotic usage is essencial to avoid unnecessary medications (FDA, 2013). Antibiotic administration should be based on the leaflet instructions and the drug manufacturer (Stanton *et al.*, 2010). Good veterinary and husbandry practices, vaccinations and controlling disease programs, should be applied

in order to decrease the need of veterinary antibiotic usage (CVO, 2014). In order to obtain more accurate and specific diagnosis results and to evaluate and control zoonotic and commensal organisms, advanced laboratory tests are required (FDA, 2013).

1.4 INCIDENCE OF VETERINARY DRUG RESIDUES

The imprudent use of antibiotics is very common especially in developed countries. A questionnaire was conducted by Yassir in 2016, to coincide the results of 122 milk samples randomly collected and tested using SNAP* Beta-Lactam ST Test and disc assay methods. It shows that 37% of samples were found to be positive for the presence of penicillin in milk (Yassir *et al.*, 2016). The results revealed the milk contamination by antibiotic residues. Moreover, different published reports confirm the presence of antibiotic residues caused by the imprudent veterinary antibiotic usage in dairy farms (El Zubeir and El Owni, 2009; Mohammed, 2011; Salman and El Nasri, 2011; Salman *et al*., 2012; Darien *et al*., 2012).

Veterinary medicine products are generally used worldwide to treat animal diseases and creating prophylactic prevention (Jacela *et al.*, 2009). In Nigeria, the detection of antimicrobial drug residues in commercial eggs (Kehinde *et al*., 2012) and in meat from slaughtered cattle (Ibrahim, 2009) is found in other studies. Other studies conducted in Ethiopia, also revealed the detection of oxytetracycline and penicillin G from milk (Desalegne *et al.*, 2014) and tetracycline from cattle beef (Addisalem *et al.*, 2019). Moreover, another study was done with 250 cattle samples that were collected from 5 slaughterhouses in and around the city of Nairobi. Chlortetracycline and oxytetracycline detection method was done using Knauer Model 128 HPLC with an electron capture detector. 114 (45.6%) samples were found positive with a mean residue levels of the detected tetracyclines that is higher than the recommended

maximum levels in edible tissues (Muriuki *et al.*, 2001). Another study in suburban and urban districts in Hanoi, Vietnam, using the agar inhibition test, and Bacillus cereus (ATCC 11778) as the reference strain with high-performance liquid chromatography (HPLC) to detect tetracycline residues, reveals that 5.5% of 290 meat samples from retail pork shops were double-positive in both tests for tetracycline residues (Duong *et al.*, 2006). In China, 7.7% of aquatic food products were found to be positive for antibiotic residue level that is unacceptable for human consumption (Hao *et al.*, 2015).

One of the biggest challenges to public health that is faced by the human population worldwide is the ongoing threat of antibiotic contamination. They are very dangerous so they can spread in food from animal origins without taking into consideration any economical, geographical and legal differences between countries (Darwish *et al*., 2013).

Moreover, in 2004, a study reported by EU revealed as well that the confirmation of the majority of residues in animals is from antibacterial agent sources (European Union, 2010).

At this time, various veterinary drugs and other environmental substances residues has also been reported in a series of working documents by the joint FAO/WHO Expert Committee on Food Additives (JECFA). In addition, evaluating the safety of residues of veterinary drugs in food and in establishing acceptable daily intakes (ADIs), and recommending maximum residue limits (MRLs) for substances when they are distributed to food-producing animals was partaking by the joint FAO/WHO Expert Committee on Food Additives (JECFA), accordingly with good veterinary practice and prudency in the use of veterinary drugs (Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2013).

Chapter 2: Concerns related to Antibiotic residues on food

2.1 CONCERNS RELATED TO HUMAN HEALTH

As declared by the United Nations in The Universal Declaration of Human Rights (UDHR) in article 25, "everyone has the right to a standard of living adequate for the health and well-being of himself; including food"; (UN, 1948). In addition, Food and Agriculture Organization (FAO) of the United Nations adopted Voluntary Guidelines in order to guide European States in offering the adequate food for the citizens. As well, FAO offer the right to the States to make sure that all food produced locally or imported, have to be checked based on national food safety standards (FAO, 2005).

Nowadays, when antibiotics are used prudently, they can reduce mortality and morbidity caused by different bacterial infectious diseases (Sanders *et al*., 2011). With the increasing of human population, the demand for food will increase, leading to an amplification of animal for food production. With the development of veterinary drugs and medicine, antibiotics cover the main interests of veterinarian, producers and farmers, (Moretain, 2005; Tatsadjieu *et al*., 2009) to use them for curative and preventive treatments or as prophylactic treatment to compensate results of animal production poor hygiene (Sanders, 2005).

Antibiotic treatment leaves residues in treated animal tissues, in other words, the food derived from treated animals to consumers (Wassenaar, 2005). The contamination of food from animal sources originated from poor veterinary practices, or/and failure to obey antibiotic pharmacovigilance by practitioner, can lead to serious health problems for consumers (Fagbamila *et al.*, 2010; Hsieh *et al.*, 2011).

Moreover, food contamination by veterinary residues and animals results from using antibiotics as growth promoters in feed (Sanders, 2005) which is nowadays prohibited in Europe (European Union, 2005_a; European Union, 2003), or by systemical administration of antibiotics for treatment purposes by a veterinarian or practitioner. When the administration does not respect the pharmacovigilance of the drug used, the drug active metabolites will remain in the treated animal tissues. This results by the presence of the administered drug residues in food produced by this animal. When contaminated food from treated animal tissue is sold into the market, food such as milk or meat will be consumed by consumers (André, 2003).

Not only, but with the development of drug-resistance in bacteria, antibiotics are often used at higher dosage than those indicated by the manufacturer recommendations (Chiesa *et al.*, 2006), such as penicillin that is used 3.5 to 10 times more than the FDA approved dose (Payne *et al.*, 2006). Furthermore, any failure in respecting antibiotic pharmacovigilance, contamination of animal feed with treated animal excretion, unlicensed and off-label antibiotic use, may result in the appearance of antibiotic residues in food originated from those animals (Paige, 1994).

Antibiotic residues in foods of animal origins above the regulatory concentration in food items established by the FDA (tolerances), EMA (MRLs), or JECFA (MRLs) may be the cause of numerous health concerns in humans. Their effect might result in either allergic reactions, acute or chronic toxicity, carcinogenicity, genotoxic, teratogenicity (when prohibited antibiotics are used and their residues are ingested in food), disruption of normal intestinal human flora in the intestine, blood dyscrasias, and/or development of antimicrobial resistance making it difficult to treat human infections (with direct or indirect contamination of the food through the animal or animal excretions). For instance, tetracycline is well used in pig farms and throughout

meat production, where residues remaining in food can affect consumers causing allergic reactions, mutagenic and teratogenic effects in some cases (Adkinson, 1980; Anonymous, 1980; 1993; 1995; Cordle, 1988; Dresser and Wilcke, 1989; Hoigné *et al.*, 1988; Pace, 1980; Riviere *et al.*, 1991).

2.1.1 **Allergy**

Immunologic reactions may manifest in many ways, from life-threatening anaphylactic reactions to lesser reactions, such as rashes. For instance, beta-lactams, that are the most problematic because many people are allergic to it (Babapour, 2012), are frequently used in veterinary medicines and are accused to causes skin allergic reaction, cutaneous eruptions, anaphylaxis and gastro-intestinal disturbance when contaminated poultry products were ingested by humans (European Union, 2018; Paige, 1997; Pene *et al*., 1988; Dayan, 1993; Punnonen *et al*., 1993; Pawankar *et al*., 1997; Gueant-Rodriguez *et al*., 2006; Mund *et al*., 2017).

Moreover, penicillin immunogenicity depends from the penicilloylation of proteins after the beta-lactam ring is open, not from the drug itself. This is why allergic reactions in humans are based on penicilloylated residues in contaminated food. Only 10 IU (0.6 mg) or 6 ng/ml of penicillin residue in milk can cause this reaction in human with sensitized history (EMA, 2008; Sundlof, 1989). Such reactions pose a risk on sensitized consumers, this is, why different competent authorities such as EMA and Codex Alimentarius established a maximum residue limit for veterinary drug, (e.g. Penicillin MRL 4 µg/ml of milk) (Codex Alimentarius, 2018).

A study was done in China between April and November 2013 in 4 schools, 1064 Han students aged between 8−11 years where tested for 18 antibiotics including five macrolides, two β-lactams, three tetracyclines, four quinolones, and four

sulfonamides residues that are commonly used in human health care or animal husbandry in China. The total detection frequency of all tested molecules was 58.3%. As for macrolides, β-lactams, tetracyclines, quinolones, and sulfonamides the general detection frequency ranged between 3.8% and 30.2%. Azithromycin, ciprofloxacin, ofloxacin, sulfamethazine, or trimethoprim were detected in more than 10% of collected urine samples. Eight of 18 antibiotics were found extremely concentrated in tested urine samples with a concentration of above 1000 ng/mL, reaching more than 40000 ng/mL for ampicillin. Of four humans and three veterinary antibiotics, the highest detection frequency was 16.4% for azithromycin and 4.2% for enrofloxacin. The overall detection frequencies of human antibiotics, veterinary antibiotics, and human/veterinary antibiotics are between 6.3 and 49.4%. (Wang *et al*., 2015b). In 1999, a 51-year-old man suffered from allergic reactions after eating some meat and salads. After the second anaphylactic shock, the sensitized man was tested with skin prick tests containing penicilloyl polylysine (Allergopen, Reinbek, Germany), a minor determinant mixture (Allergopen), benzylpenicillin at 25 000 UI/ml, ampicillin, amoxicillin at 25 mg/ml, and ceftriaxone and cefapirine at 25 mg/ml (all diluted in normal saline). Allergic reactions were totally visible on the patient skin to all drugs used, and at the same time, the patient experienced another anaphylactic shock. Three months later, the sensitized man developed contact urticaria that was generalized rapidly with conjunctivitis fifteen minutes after dripping one drop of amoxicillin at 25 mg/ml on the skin of his forearm, without cutaneous breaking (Dayan, 1993). Such cases are reported frequently. For instance, a lady of 64 years old that survived 4 anaphylactic reactions where 2 of them after pork and beef ingestion, and it was confirmed by a positive prick test to penicillin G 1IU/ml, that it was a reaction to that

veterinary drug. At a cumulative dose of 20 IU in milk, wheezing and hypotension where seen as well (Kanny *et al*., 1994).

Not only adult consumers, but as well, the incidence of allergic reactions in newborns, children aged 1 to 2 and 3 to 10 years are respectively, 5%, 11% and 9% (Gill, *et al*., 1995). Translating those results into another way, the incidence of allergic reactions is about 76% with children aged less than 7 years (Graff-Lonnevig *et al*., 1988) and 59.5% for those aged between 7 and 12 years old (Venuti *et al*., 1992). Sometimes the incidence of neonate's allergic reaction can reach 24.5% showing erythematous maculopapular rashes in 67.1% of the cases (Kushwaha *et al*., 1994). Antibiotics are the main molecules that causes those high numbers of allergic reactions (Boguniewicz and Leung, 1995; Lewis *et al*., 2001; Weiss *et al*., 2002; Wilson, 1995) and as usual, beta-lactams are on the top of the list of those allergies (Erffmeyer, 1992), with an average between 55.2 % to 67.7 % for children (Kamada *et al*., 1991; Romano *et al*., 1993). Not only beta-lactams, but also aminoglycosides, tetracyclines and macrolides show allergic reaction in children (Romano *et al*., 1993).

2.1.2 **Toxic effect**

Acute toxicity effect is related to a high dose of antibiotic residues that is consumed while ingesting contaminated food. But when ingesting small doses of antibiotic residues while consuming the same food that is contaminated with the same antibiotic, this might lead to chronic toxicity. A study was done in 1989, estimating violations of antibiotic class administration. Streptomycin, penicillin, tetracycline, gentamicin, sulfamethazine and neomycin were abused 705 times by the route if administration that was recommended by the manufacturer and 460 (60%) of those violations were caused by intramuscular administration (Guest and Paige, 1991).

When antibiotics are injected intramuscularly or sub-cutaneously, residues will remain at the site of injection more than when administered orally, and the potential of consumer contamination by ingesting the injection site is higher.

A study was done in France on 5 Friesian dairy cows (aged between 4 and 7 years) and 9 cross bullocks (weighing between 200 and 250 kg). In this study, cows were intramuscularly injected every 12 hours by a combination of chloramphenicol and oxytetracycline for 3 days. 14 days after treatments, no residues were found in milk or edible tissues but, at the site of injection, chloramphenicol and oxytetracycline were detected 21 days and 35 days respectively post injections (Guillot *et al*., 1989).

Another study was done at the University of Saskatchewan Beef Research Centre using 65 healthy crossbred yearling beef steers weighing an average of 485 kg. The study was aimed to check penicillin G with procaine penicillin G concentrations at the site of injections with respecting of withdrawal period directed by the drug manufacturer. Administered dosage of benzathine penicillin G with procaine penicillin G at recommended levels (intramuscularly at 8,600 IU/kg in Canada, or subcutaneously at 8,800 IU/kg in the USA), do not show any residues exceeding MRL (50 μg/kg) after the withdrawal periods (14 days in Canada, 30 days in the USA) in all tested tissues, but at the injection sites, antibiotic residues were exceeding the MRL by 30 to 60 time.

When only benzathine penicillin G was intramuscularly injected at 24,000 IU/kg, no residues were detected in muscles 8 days post injection and at 14 days post injection, residues were below MRL in kidneys and liver but 50 days post injection, residues were 24 times over the MRL at the site of injection.

When benzathine penicillin G with procaine penicillin G were intramuscularly injected at 12,000IU/kg, no residues were detected in muscles and kidneys and

residues found in the liver was below the MRL, but at the site of injection, residues were found to be 156 times higher than MRL (Korsrud *et al*., 1993).

FDA shows in her 103 FDA Center for Veterinary Medicine CVM records of residues violations with route of administration, that the big number of those routes were injections 51.3% from FDA CVM and 61.7% from Virginia Department of Agriculture and Consumer Services (VDACS), following with feed supplementation with 21.4% from FDA CVM and 6.7% from VDACS and bolus with 15.4% from FDA CVM and 26.7% from VDACS (Van Dresser and Milcke 1989). Toxicity effect caused by antibiotic residues is related to the dose of residues ingested. For instance, acute toxicity can result after the ingestion of a peace of meat that was used as site of antibiotic injection, or when consuming milk collected from an udder that was treated with intra-mammary antibiotic tubes and in both cases withdrawal period was not respected.

Another study was performed on ICR mice in Jilin University in order to clarify the toxic effects of Benzylpenicilloic acid (BPNLA). Acute oral and intraperitoneal toxicity analysis were done in mice for 14 days, and the results show that the lethal acute toxicity of BPNLA for oral dose administration is greater than 200mg/kg. Concerning intraperitoneal injections, 12.44g/kg of BPNLA were found to show lethal effect in 1 mouse, 1 hour after injection to reach a mortality of 100% of injected mice. BPNLA LD50 value of acute intraperitoneal toxicity was 8.48 g/kg with 95% confidence interval between 7.76 g/kg to 9.27 g/kg) (Cheng, *et al.*, 2017).

Field experience defer from laboratory findings. For instance, based on data in New Zealand, injection site residues might be ingested once every 1.8 to 45 years (Brown, 2000). Moreover, in Europe, using a probabilistic approach, the estimation that the maximal likelihood of a consumer ingesting an injection site, or a part of it,

was four times annually, while in the same period, 37% of European consumers would not ingest an injection site. (Sanquer *et al.,* 2006). keeping in mind that such estimations are necessary, they take into consideration a series of worst-case scenarios that are unlikely to happen in daily life (Galer & Monro, 1996).

On another level, chronic toxicity to antibiotic residues may occur when consumer is consuming the same residue daily for a long period of time.

A 6-month study was done on 80 ICR mice in Jilin University to study the effect of chronic administration of benzylpenicillin G residues heated to cooking temperature (BPHCT) on different essential factors. Results show that 10% of tested mouse were found crawling in circles after 2 months of BPHCT oral administration for 2 weeks in the group of 60 (925 μg 25 g-1 day-1) and for the last of the study in the group of 600 (9.25 mg 25 g-1 day-1). No dead animals were found at the end of this study but the body weight were affected negatively in females and the group of 600 males, and positively in the male group of 6 (92.5 μg 25 g-1 day-1) and 60. Concerning organs toxicity, 30% of mouse in the 600 dose group shows liver, lungs and testicules negative affection. In the liver, diffusely hepatocytes showed eosinophilic granular cytoplasm, individual hepatocyte, necrosis, lymphocytic and histiocytic inflammatory infiltrate in the liver parenchyma. The pulmonary changes consisted of multifocal lymphocytic, histiocytic and plasmacytic inflammatory infiltration in the parenchyma. Furthermore, in the testis, seminiferous tubules were absent or fewer cellular than the control cases, while the interstitium was broadened. There was diffuse severe necrosis of the spermatogenic lineage cells, decreased number of sustentacular cells and spermatids were absent or infrequent (Cui, *et al.*, 2018).

A lot of studies using animal laboratories shows that the incidence of chronic toxicity exists, but in real life, taking into consideration a daily food basket that is

constituted of cereals, bread and pasta 40%; 2. sugar and fat 1-2%; 3. meat, fish and eggs (12,5%); 4. milk and dairy products (12,5%); 5. fruits (14%) and 6. Vegetables (20%) (Arnaut-Berilo, *et al*., 2017) shows that the real risk of chronic toxicity is not present, unless the same consumer is consuming the same food that is contaminated with the same antibiotic residue with enough concentration that remains from a meal to another, for a minimum of 6 months. The true risk of chronic toxicity is almost impossible, but the potential option of chronic toxicity is everywhere, especially in developing countries were lack of residue control affect consumer's health indirectly.

2.1.3 **carcinogenicity, genotoxic and teratogenicity**

Carcinogen term is used to an effect produced by a substance playing carcinogenic action (ACS, 2014). The possibility of having this carcinogenic effect is related to the exposure frequency to carcinogenic residues and their interactions with different intracellular constituents such as proteins, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), glycogen, phospholipids, and glutathione (Aiello *et al.*, 2005).

Mutagen term is related to any chemical or physical agents such as drugs and environmental chemicals with the potential of causing a mutation in human or animal DNA molecules or damages in the genetic constituent of their organism or cells (Booth and Mc Donald, 1988; Foster and Beecroft, 2014).

Teratogen term is related to any drug or chemical agent that can affect an embryo or a foetus during gestation. Congenital malformation affecting the structure and the function of the organism might be the results (Booth and Mc Donald, 1988; Beyene, 2016).

In order to prevent any consumer's health problems resulted from veterinary drugs that are used in animals for food production, Competent Authorities in

developed countries such as European Union and USA, have enforced the prohibition of drugs that might cause any human risk. In order to assure prudent antibiotic use, Competent Authorities have established different associations such as FDA, CAC, EFSA, European Council, Food Animal Residue Avoidance Databank (FARAD) and others to manage the use of such drugs in animal for food production. Drugs classification is listed in the Codex Alimentarius, European Council, FARAD, FDA, and EFSA, but only some of them are antibiotics for veterinary use (FDA, 2018; Codex Alimentarius, 2018; EFSA, 2014; European Union, 2010; European Union, 1990; FARAD, 2018).

Chloramphenicol is an old antibiotic that was approved for human use by FDA in 1950 and was prohibited for the use in animals for food production (FDA, 2012a). This antibiotic is a broad spectrum gram negative and positive bacteria, with a wide distribution mechanism that can reach most of the treated body tissues and fluids including central nervous system, placenta and mammary glands (Riviere and Papich, 2009). On the other hand, adverse effect can result from chloramphenicol residues in human, most significantly, bone marrow suppression leading to aplastic anemia (Eliakim-Raz *et al.*, 2015). The induction of aplastic anemia is not dose dependent (Wongtavatchai *et al.*, 2004). People such as, veterinarians, animal handlers and drug handler practitioners that are in contact with chloramphenicol are at risk, because a small exposure might be the reason of aplastic anemia appearance. For instance, in 1981, a feed lot rancher has passed away from aplastic anemia caused by chloramphenicol that was in contact with an open wound on his hand while treating cattle that were affected by pneumonia (Settepani, 1984).

Chloramphenicol is classified as genotoxic and a possible carcinogen by JECFA and WHO (FAO/WHO, 2004). Moreover, it is not well known yet, this is why it is not

classified between drugs (Codex Alimentarius, 2018). EFSA has identified the presence of chloramphenicol residues in different food from animal origins such as milk, meat, and others after therapeutical chloramphenicol use (EFSA, 2014). The acceptable daily intake of chloramphenicol is not determined yet. Few information are available in order to establish the minimum dose that could launch aplastic anemia, carcinogenicity and reproductive toxicity of this drug residues (JECFA, 1994; Wongtavatchai *et al*., 2004). A study was done in Taiwan in order to monitor results for commercial livestock products between 2011 and 2015. Results in table 3 of this study shows that chloramphenicol residues were found to be positive in pork meat from 2011 until 2015 (Hsin-Chun *et al*., 2017). There is no approved evidence to confirm that chloramphenicol residues in food are causing hazardous problems to consumers, but different studies have reported many health problems in humans that were treated with chloramphenicol. For instance, in California 1969, patients treated with chloramphenicol are 13 time more susceptible to aplastic anemia than the general population, knowing that the majority of treated patients were between 50 and 80 years old, but 2 exceptions took place with a 15 years old boy treated with a total of 3 g of chloramphenicol and a 37 years old female treated with a total of 6 g of chloramphenicol over a month. In both cases the onset of aplastic anemia occurred 3 to 4-month post-treatment (Wallerstein *et al*., 1969). In addition, in Istanbul, 4 of 108 aplastic anemia cases were caused by chloramphenicol treatment (Aksoy *et al*., 1984). Moreover, a 27 years old female was treated with 30 g of chloramphenicol intravenously for 12 days, develops aplastic anemia 3-month post-treatment (Alavi, 1983). Another 26 years old pregnant woman shows anemia and skin infection symptoms at the 5th and 6th month of pregnancy. She was treated with a total of 8g chloramphenicol, and developed aplastic anemia. Her death occurred 8 days after

giving birth and aplastic anemia was confirmed by bone marrow aspiration (Suda *et al*., 1978). In Shanghai, 641 patients treated with chloramphenicol showed blood dyscrasias and 464 were identified as aplastic anemia and 27 cases as leukemia (Shu *et al*., 1987).

Not only chloramphenicol, but also nitrofurans a broad-spectrum antimicrobial drug used for therapeutical and prophylactic purposes in food producing animals. Because of the insufficient information related to mutagenicity and carcinogenicity of nitrofurans, no Acceptable Daily Intake (ADI) was established and it was banned for veterinary medicine use in food producing animals (EMA, $2009_{b;}$ FDA, 2018; Codex Alimentarius, 2018; EFSA, 2014; European Union, 2010; EEC, 1990; FARAD, 2018). As requested from the Codex Alimentarius, JECFA has evaluated nitrofurazone during the 40th meeting in 1992 (WHO, 1993_b and FAO, 1993). Nitrofurazone showed acute toxicity in lung during laboratories experiments on animals. Symptoms were clear, showing a decrease in respiratory function and death. Moreover, neurotoxicity such as hyperirritability, tremors and convulsions were found as well (EFSA, 2015). Not only, but nitrofurazone causes liver, kidneys and testes toxicity, decrease in the weight gain and neurotoxicity with 13.5 mg/kg body weight per day (Highest dose at which there was not an observed toxic or adverse effect; NOAEL) for testes toxicity in rats and mice. Moreover, when administered orally for mice and rats, nitrofurazone improves the occurrence of benign tumors in endocrine organs and mammary glands with an increase of preputial glands carcinoma in male rats (EFSA, 2015_a).

Furazolidone is another antibiotic drug that can be used for veterinary therapeutic and preventive purposes in animals for food production. Due to its carcinogenicity and genotoxicity, Competent Authorities has forbidden its use in animals for food production (EMA, $2009_{a;}$ FDA, 2018; Codex Alimentarius, 2018;

EFSA, 2014; European Union, 2010; European Union, 1990; FARAD, 2018). Moreover, no ADI for this forbidden drug and its metabolites in food could be acceptable for consumers (WHO, 1993$_b$). Acute toxicity research's demonstrates, using laboratory animals, that furazolidone can cause lugs toxicity by decreasing respiratory function and death caused by asphyxia (EFSA, 2015$_b$). Moreover, neurotoxicity such as hyperirritability, tremors and convulsions were clearly visible with testes toxicity in male rats. In addition, furazolidone is embryotoxic in mice at a minimum dose of 200 mg/ kg body weight per day with decreased body weight and viability in newborns (EFSA, 2015).

In furazolidone chronic toxicity and carcinogenicity laboratory studies, malignant mammary tumors in rats, bronchial adenocarcinomas in male and female mice and neural astrocytomas in male rats are found (EFSA, 2015). Based on the previous listed findings, EFSA Scientific Panel on Contaminants in the Food Chain (CONTAM Panel) concludes that furazolidone is carcinogenic in mice and rats pausing an unclear hazard and a risk on human health (EFSA, 2015).

Stilbenes and carbadox are other drugs that are used in food producing animals as growth promoters for the improvement of weight gain and feed efficiency. Because they and their metabolites have no safe level of residues, their use in food producing animals is prohibited by the Competent Authorities (EMA, 2009a; Codex Alimentarius, 2018; CCRVDF, 2012; EFSA, 2014; European Union, 2010; European Union, 1990; FARAD, 2018).

International agency for research on cancer has stated that stilbenes metabolite is carcinogenic to humans (group 1), with clear linked exposure to stilbenes metabolites with cell adenoma in women's vagina and cervix that were exposed to this metabolite in utero. Moreover, women that were exposed to stilbenes metabolites

during pregnancy shows mammary cancer. Additionally, there is a clear association between endometrium cancer, testes cancer and squamous cell carcinoma of the cervix with stilbenes metabolites exposure. In female mice, high prevalence of ovarian, endometrial and cervical tumors, as well as mammary adenocarcinomas were caused by stilbenes metabolites exposure. Furthermore, incidence of osteosarcomas and Leydig cell tumors were amplified when respectively male mice (rasH2 and XPa/p53) were exposed to stilbenes metabolites (Codex Alimentarius, 2012).

Carbadox was proved to be genotoxic and hepato-carcinogenic in rats (JECFA, 2003). But the metabolites found in animal tissues quinoxaline-2-carboxylic acid (QCA) were not carcinogenic or mutagenic. Nowadays old MRLs are withddrawn because the Committee could not determine the amounts of residues of carbadox and its metabolites in food that represented an acceptable risk to consumers (JECFA, 2003).

2.1.4 **Negative effect of antibiotic residues on consumer's gut microflora**

Humans could be exposed daily to veterinary drug residues throughout ingesting contaminated food. The outcomes caused to intestinal microflora following acute active residues intake is different from when residues are ingested chronically (Cerniglia *et al.*, 2016). Acute intake is when food ingested contains one single residue dose and the contaminated food is ingested as one meal time event then transit all over the gastrointestinal tract reaching the colon that contains only the ingested drug residue. When chronic exposure to active drug residue take place, the event of daily contaminated food ingestion with the same residue should occur; everyday, ingested contaminated meal reach the gastrointestinal system that already contains residues of the same ingested drug over a lifetime (JECFA, 2017). This is why, the frequency of

microflora exposure to an ingested drug residue in vivo from acute dose is lower than when chronic ingestion occurs (JECFA, 2017). Moreover, there is a sequence how ingested residues are transported through the esophagus, stomach, small and large intestines which is provided by transit and peristaltic movements, leading to the appearance of residues as small doses over time in the intestines lumen (Pišlar *et al*., 2015). A study was done using radioactive pellets and fiber in order to monitor their passage in healthy volunteers. Results support the previously described transit and peristaltic mechanism of the gastrointestinal tract and show that radiolabel was distributed at the same moment in the colon, stomach, and small bowel with 10, 20 and 70 percent respectively, proving that a contaminated meal will not transit as a single bolus dose (Camilleri *et al.*, 1989).

Different outcomes of the risk assessment on the impact of residues of antibiotic on gut microflora occurred considering ingestion at therapeutic dose or at residual level.

Tetracycline is used in some cases to treat humans with therapeutic doses that might affect patient microflora. For instance, a short-term stable tetracycline dose of 12.5 mg/kg and 25 mg/kg in small and large human intestines respectively, does not affect their gut microflora composition (Burton *et al.*, 1974; Corpet *et al*., 1989). Old studies showed that human treated with oxytetracycline at a dose of 10 mg/day for 6 months have built a high number of oxytetracycline resistant coliforms and yeasts (Goldberg *et al.*, 1961).

In addition, humans treated for acne vulgaris with 100 mg/day of tetracycline for a long period of time shows a change in the resistance levels of microflora by increasing the percentage of transferable R-factor bacteria in humans as well as the number of multi-resistant strains (Valtonen *et al*., 1976).

A study was done on healthy volunteers by giving them for 4 days 0, 50, or 1000 mg/d of tetracycline to understand the effect of tetracycline therapeutic dose on human. Results show that 50 mg/day of tetracycline does not affect human microflora but increases tetracycline resistant *E. coli* shedding from the intestine when receiving orally 1000 mg of tetracycline/day (Hirsh *et al*., 1974).

Different studies have showed that when antibiotic are administered for treatments purposes in pediatric or adult infections affects negatively treated human microbiome in terms of modifying microbiota.

The following antibiotics have showed this effect in different research:

Amoxicillin (Gipponi *et al.*, 1985), Ampicillin (Greenwood *et al*., 2014; Hernandez *et al.*, 2013; Maurice *et al.*, 2012), Cefotaxime (Sunakawa *et al*., 1984; Lambert-Zechovsky *et al.*, 1985;) Chloramphenicol (Maurice *et al.*, 2012) Ciprofloxacin (Brismar *et al*., 1990; Dethlefsen *et al*., 2008; Dethlefsen and Relman, 2011; Maurice *et al*., 2012) Clarithromycin plus metronidazole (Jakobsson *et al.*, 2010; Clindamycin (Kager *et al*., 1981; Jernberg *et al*., 2007; Zaura *et al*., 2015; Lichtman *et al*., 2016; Jump *et al*., 2014), Erythromycin (Brismar, *et al*., 1991; Maurice *et al*., 2012), Gentamicin (Greenwood *et al*., 2014, Zhao *et al*., 2013) Meropenem (Bergan *et al*., 1991) Streptomycin (Lichtman *et al*., 2016; Antunes *et al*., 2011), Ticarcillin (Nord *et al*., 1989), Tigecycline (Nord *et al*., 2006; Bassis *et al*., 2014) Vancomycin (Vrieze *et al*., 2014; Maurice *et al*., 2012; Morgun *et al*., 2015; Yap *et al*., 2008; Antunes *et al*., 2011; Cho *et al*., 2012).

Furthermore, a study was performed on three healthy humans to investigate the distal gut bacterial communities before and after ciprofloxacin treatment. The study shows that antibiotic treatment has affected the abundance of around third of 30% of the bacteria found in the gut, decreasing heavily taxonomic richness, diversity and

uniformity. Moreover, some taxa failed to recover within 6 months (Dethlefsen *et al.*, 2008)

Another study was performed on three healthy volunteers for 10-month period in which they received 2 courses of ciprofloxacin. The experimental study analyzed 1.7 million bacterial 16S rRNA hypervariable region sequences from 52 to 56 samples per volunteer. Results shows that ciprofloxacin effect was fast and deep and affected the diversity and the community composition happening within 3 to 4 days of initiating the drug. Furthermore, after the end of the experiment, none of the communities could return to the initial situation (Dethlefsen and Relman, 2011).

On a residual level, few studies on the effect of the residual level of antibiotic on human's gut microbiota are available. But some of them prove that common antibiotics between animals and human have dangerous effects when ingested. A study concerning the effect of tetracycline on human cell was done to show the negative effect of residues on human cells. T84 cells (ATCC® CCL-248™) was used, a human colorectal carcinoma cell line, obtained from ATCC (Manassas, VA). Cells culture was performed using complete growth media, which was composed of Dulbecco's Modified Eagle Medium (DMEM)/F-12 medium supplemented with L-glutamine and [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] HEPES (ATCC), with added 5% fetal bovine serum, penicillin/streptomycin, and fungizone.

It is well known that the epithelial layer in the intestinal lumen system has a biochemical barrier role between host and microbes (Odenwald and Turner, 2013; Pastorelli *et al.*, 2013). Experimental results show that cells treated with a concentration of 150 mg/ml tetracycline could not migrate to heal an injured epithelial surface layer (Kuppan *et al*., 2017).

In Kuppan's study done in 2017 in order to evaluate whether residual concentrations of tetracycline impact epithelial cell integrity, intestinal cells were affected by 15 and 150 mg/ml of tetracycline 24 and 48 hours after the 1st antibiotic-cell contact, indicating a perturbation in the intestinal barrier defense mechanism as well as gap junctions that are imperative for cells barrier integrity, cellular function and gut homeostasis.Ggene expression was affected as well at 1.5 µg/ml residual level of tetracycline (Kuppan *et al.*, 2017).

Another research was done on 6 volunteers using tetracycline at 2 and 20 mg/day for 7 consecutive days, to study tetracycline residues effect on human intestinal microflora. Result shows that subjects receiving 2mg/day had no alteration on the total *E. coli* even with the presence of tetracycline in feces, but when 20 mg/day was administered, intestinal microflora was affected (Tancrede and Barakat, 1989).

Another study was done on 60 C57BL/6J mice in order to compare the multiplication of total microbiota. Mice were exposed to 50 µg/kg of ampicillin, 100 µg/kg of tetracycline or 100 µg/kg of sulphadiazine. Results have proved that the residues of antibiotic in foods even at very low levels can disturb mouse gut microbiota by increasing Proteobacteria about 2 logs CFU/g and decrease Bifidobacterium and Lactobacillus about 1 log CFU/g in case of ampicillin and sulphadiazine (Roca-Saavedra *et al.*, 2018).

Furthermore, a study done by Mumtaz proved that ingested chicken meat contaminated with antibiotic residues may affect consumer's microflora (Mumtaz *et al.*, 2000).

2.1.5 **Role of residues in antimicrobial resistance spreading**

The first antimicrobial resistance was found in 1940 with Bacillus coli that is now recognized as *Escherichia coli* (Chain *et al.*, 1940; Abraham and Chain, 1940). An evolutionary tree uncomplicated the origins of resistance, proving that bacteria evolved antimicrobial resistance genes long before the antibiotic epoch (Benveniste and Davies, 1973; Finley *et al.*, 2013, Aminov and Mackie, 2007; Wellington *et al.*, 2013; Martinez and Baquero, 2009). Moreover, some of them have developed defenses mechanisms against synthetic compounds (D'Costa *et al.*, 2011). Antimicrobial resistance is an old natural characteristic of the environmental bacteria genome (Bhullar *et al.*, 2012). It is important to take into consideration the low incidence rate of antimicrobial resistance before antibiotic usage era, and their increase after lunching antibiotics in market, proving that antibiotic usage is an important reason for antimicrobial resistance (Hughes and Datta, 1983).

Bacteria can have natural resistance to some antimicrobials which is called intrinsic resistance (Prescott, 2008), or they can build up resistance through different biological changes such as mutations and this process is called acquired resistance (FAO, 2016).

Intrinsic resistance depends on chromosomal genes of the bacteria (Alekshun and Levy, 2007, Courvalin, 2008) that is usually linked to the anatomical physiognomics of it (IFT 2006), which is a shared characteristic between organisms having the same species or genus (Courvalin, 2008). For instance, penicillin G resistance is usually a form of Gram-negative bacteria resistance (Boerlin and White, 2013; Scenihr, 2009), due to its mode of action by inhibiting the proteins which cross-link peptidoglycans in the gram positive cell wall bacteria (Amasino *et al.*, 2006).

Acquired resistance could happen in vertical and horizontal transmission. Vertical transmission is caused by chromosomal mutations that are extremely rare with a frequency of 10^{-7} to 10^{-9}. It is usually related to the development of resistance in bacterial clones (Courvalin, 2008). This mutation can affect regulatory, or target genes that encodes the specific role of antimicrobial action (Courvalin, 2008). When an antibiotic is introduced, single point mutations are usually detected, similarly to the mutation that occurred with quinolone and macrolide resistance in *Campylobacter spp.* (Aarestrup *et al.*, 2008; Moore *et al.*, 2006, Cambau and Guillard, 2012). Regulatory mutation is usually spontaneously and affect the gene expression mechanisms (Courvalin, 2008).

Horizontal gene transmission is when a cell transfers her genes to another cell independently from a reproductive event. It is most probably the main mechanism of bacterial resistance, helping bacterial population to spread and increase resistance emergence (Aarestrup *et al.*, 2008).

This type of transmission can occur through 3 main mechanisms:

1) Transformation which is an uptake of a free DNA by a competent bacterial cell.
2) Transduction, which is the movement of a bacterial DNA from a bacterial cell to another by bacteriophages.
3) Conjugation, which is the movement of a bacterial DNA from a donor bacterium to a recipient bacterium by physical contact and conjugative machinery (Amábile-Cuevas and Chicurel, 1992; Amábile-Cuevas, 2012).

Five different mechanisms help the bacteria to become resistant to one or different antimicrobials and they are classified into the following: (Van Hoek *et al.*, 2011).

1. Decrease of the antimicrobial accumulation inside the cell. This could be done through reducing permeability and/or activate efflux of the drug from the bacterial cell.
2. Degradation or modification of the antibiotic by bacterial enzymes.
3. Attainment of substituted metabolic pathways to those blocked by the antibiotics.
4. Protection or modification of antimicrobial target.
5. Extra-production of the targeted enzyme.

2.2 BACTERIAL RESISTANCE SPREADING MECHANISM IN THE ENVIRONMENT

Pathogenic and non-pathogenic resistant bacteria have the possibility to be transmitted from livestock to humans through consumed food from animal origin, direct contact (while working with animals) and their waist (when used in the environment) (Marshall and Levy, 2011). Fomites as well play a major role in bacterial resistance spreading. For instance, in Denmark, a multidrug-resistant *Salmonella enterica* serovar typhimurium DT204 was deeply studied and farm equipment were found to be an important vector of bacterial resistance transmission (Aarestrup, 2006). Knowing that bacteria can share their genetic elements with other bacteria from the same or different strain in any environment that enhance their mixture such as human or animal gut, aquatic environment, slurry spread or agricultural soil, it is important to keep in mind that any single mechanism that can promote bacterial transmission, can as well increase the spreading of resistant bacteria (Woolridge, 2012, Aarestrup, 2006, Baquero *et al.*, 2008). For instance, if environmental bacteria develop antibiotic resistance, humans and animals will be at risk, especially when water, food crops and animal feed will become infected creating an opportunity to share resistant

mechanisms between commensal and pathogenic bacteria found in animal and human guts (Aarestrup, 2006, Finley *et al*., 2013, Marti *et al*., 2013).

Antibiotic used orally in livestock can be frequently excreted in feces affecting bacterial population in soil and water (Woolridge, 2012, AAM, 2009) as well as residues excreted from humans treated with antibiotics can exert selection pressure on environmental bacteria (Igbinosa *et al*., 2011; Baquero *et al.*, 2008; Finley *et al*., 2013; Wellington *et al*., 2013; Novo *et al*., 2013). With all what we know about bacteria and their resistance, there is not enough evidence to understand resistance transferring mechanisms (Hong *et al.*, 2011; McEwen, 2006; Novo *et al*., 2013; Woolhouse *et al.*, 2015).

Water, one of the life basic elements, can be used directly by humans and animals or indirectly through irrigating crops that will be consumed later (Finley *et al*., 2013). It plays a big role in spreading resistant bacteria and antibiotic residues especially in low-/middle-income countries (LMICs) where water has been the major vector of pathogenic bacteria transmission to human (Wellington *et al.*, 2013). Moreover, antimicrobial resistant bacteria have also been detected in recreational water in a study done in England and Wales (Leonard *et al*., 2015). Drug factories play a big role in spreading bacterial resistance through their wastes especially in low cost manufacturing countries (Larsson *et al*., 2007; Sim *et al.*, 2011; Mutiyar and Mittal, 2014; O'Neill, 2015). For instance, in highly populated Asian countries, such as China, India, Bangladesh and Pakistan, pharmaceutical pollution is described as "serious threats to the environment". A study in those countries showed that almost all of the inspected pharmaceutical industries are discharging their waste without any treatments into domestic sewage networks, promoting antibiotic resistance that can affect humans and animals via aerosols, endophytes, water and crops (Rehman *et al*., 2013; epha,

2015). Another study was done by Swedish scientist's team in 2007 in order to investigate water pollution in India, Hyderabad, specifically in areas surrounding pharmaceutical industries. Patancheru industrial zone was extremely emitting polluted wastes to the point that some water samples were contaminated with antibiotic residues with a higher concentration than those found in a medicated patient blood (Larsson *et al*., 2007). Moreover, ciprofloxacin concentration was around one million times greater than those normally found in public sewage waste with a high range of toxicity. This quantity of ciprofloxacin was around 44 kg per day, that is enough to treat a city of 44,000 citizens, or can be used in Sweden for 5 days (Larsson, et al., 2007). Other studies showed that river sediment, waste (Kristiansson *et al*., 2011), soils (Rutgersson *et al.*, 2014), surface, ground and drinking water (Fick *et al*., 2009) are polluted for the first time by pharmaceutical to such extremely level (epha, 2015).

Another essential element for life is air, which can play an important role for the spreading of antibiotic residues, antimicrobial resistant genes and bacteria (McEachran *et al.*, 2015). The mechanism of transmission needs areas that are prone to soil scouring, dust formation and windy weather, similar to some areas in the United States where large cattle feedlots are available. People living around are highly exposed to antimicrobial resistant bacteria, residues and resistant genes via direct inhalation of contaminated dust, deposition of resistant bacteria or residues on the skin or by ingesting contaminated food and water (McEachran *et al*., 2015).

It is unclear if the antibiotic residues and antimicrobials resistant genes originated from livestock or human sources are dependent, knowing that environmental transmission pathways by livestock contaminants still remain imprecise (AAM 2009; Wellington *et al*., 2013; Marti *et al*., 2013). Many gaps in nowadays information, because environmental sites are unstable and might be affected by

different natural events such as watercourses that are dynamic with water diluting effect (Woolridge, 2012).

The link between human sources of environmental contaminant and livestock production in correlation with the spreading of environmental antimicrobial resistance is yet undetermined. While worldwide, different studies have related as a hypothesis, the occurrence of resistant genes in the environment resulting from livestock and aquaculture wastes contamination (Woolridge, 2012; Binh *et al*., 2007; Acar and Moulin, 2006; Zhao *et al*., 2010; Hong *et al*., 2011; Heuer *et al*., 2002; Heuer and Smalla, 2007; Quintana-Hayashi and Thakur, 2012; Li *et al.,* 2012).

2.3 BACTERIAL RESISTANCE SPREADING MECHANISM VIA FOOD DISTRIBUTION

Food born transmission is becoming the main interest in order to understand the likelihood of livestock-human antimicrobial resistance spreading (Woolridge, 2012). When food is contaminated by resistant bacteria, it will become a direct vector for consumer's infection (Hong *et al*., 2011; Marti *et al*., 2013). Moreover, in quantitative terms, the most important way for antimicrobial resistance transmission from livestock to humans is through food ingestion, keeping in mind that environmental transmission to which humans are frequently exposed, is still not totally understood (Capita and Alonso-Calleja, 2013).

Meat contamination can be an important source of antibiotic resistant bacteria transmission. Different studies revealed the presence of antibiotic resistant bacteria are common between animals and humans. For instance, in Kenya, *E. coli* isolated form beef samples found in the market, shows resistance to ampicillin (31 %), tetracycline (20 %) and nalidixic acid and ceftazidime (4 %) and 27 % of those isolates shows

multidrug resistance (Kariuki et al., 2013). Moreover, in the United States, The National Antimicrobial Resistance Monitoring System (NARMS) have published a comparative study to show the percentage of resistant *Salmonella*, *E. coli*, *Enterococcus* and *Campylobacter* in 2015 from a big number of isolates to different antibiotics that are common for humans and animals (table 2 and 3) (NARMS, 2017$_c$).

Table 2: Showing the percentage of *Salmonella* isolates resistant to different antibiotics in 2015 in USA (NARMS, 2017c)

Antibiotic	Human	Retail Chicken	Retail ground turkey	Retail ground beef	Retail pork shop
Amoxicillin & Clavulanic Acid	2.7	13.1	4.1	16.7	0
Ampicillin	12.4	15.6	20	16.7	21.1
Azithromicin	0.3	0	0.7	/	/
Cefoxitin	2.5	11.8	4.1	16.7	0
Ceftiofure	2.7	12.2	4.8	16.7	0
Ceftriaxone	2.7	12.7	4.8	16.7	0
Chloramphenicol	3.3	0	0	33.3	5.3
Ciprofloxacin	0.4	0	0	/	0
Decreased suceptibiity to Ciprofloxacin	5.8	0	0	0	5.3
Gentamycin	1.8	4.2	22.1	0	5.3
Nalidixic Acid	4.7	0	0	0	0
Streptomycin	15.5	30.8	47.6	33.3	36.8
Slufamethoxazole-Sulfisoxazole	11.8	27	29	33.3	26.3
Tetracycline	13.5	47.3	24.1	50	47.4
Trimethoprim-Sulfamethoxazole	2.4	0.4	0.7	16.7	0

Table 3: Showing the percentage of *Campylobacter* isolates resistant to different antibiotics in 2015 in USA (NARMS, 2017c)

Antibiotic	Human	Retail Chicken	Retail ground turkey
Azithromicin	3.6	5.2	20
Chloramphenicol	3	/	/
Ciprofloxacin	25.8	18.5	40
Clindamycin	4.5	4.9	20
Erythromycin	3.6	5	20
Florfenicol	1.5	0	/
Gentamycin	1.9	1.7	40
Nalidixic Acid	25.8	18.7	40
Telithromycin	4.8	4.7	20
Tetracycline	45.6	44.7	80

People traveling between developed and developing countries as well as food trading, play a role in antimicrobial resistance spreading and this was proved in a number of studies where meat (Skov *et al*., 2007), chicken (Wilson, 2003; Warren *et al*., 2008) fish (Ozawa *et al*., 2002l; Noor Uddin *et al*., 2013) and dairy products were carriers for antibiotic resistant bacteria (Zhao *et al*., 2003; Hong *et al*., 2011). With the increase of population, food intake is increasing and spreading as well, especially with nowadays globalization and transportation technologies that made worldwide food shipping easier (Aarestrup, 2006). For pay off, resistant bacteria in shipped food, can in a short period of time, reach new areas and infect them (Okeke *et al*., 2005).

A study done in 3 feedlots in Nebraska, shows that a third-generation cephalosporin and trimethoprim-sulfamethoxazole-resistant *E. coli* was found in 100%

of tested animals, but only 0.5% were found on carcasses and 0% on retail meat derived from the same animals (Schmidt, *et al*., 2015). In Denmark 1990s, concerns about avoparcin use as growth promoter in livestock were present because of some proof of a link to vancomycin resistance in humans, knowing that vancomycin use in European hospitals was very low but with a high level of resistance in humans (Wielinga *et al*., 2014). Another study in Netherlands investigated vancomycin–resistant enterococci in feces of people that have been vegetarians from 3 to 86 years, and meat eaters that were living in separated houses. Enterococci selective procedure was used and shows 10% of vancomycin–resistant *E. faecium* in meat eaters but 0% in vegetarians (Schouten *et al*., 1997). In South Western Nigeria, a study was done in order to find streptomycin residues in goat, cattle and pig meat sold for human consumption. Results show that 17.22%, 16.11% and 6.67% of samples from goat, cattle and pigs respectively, were positive for residues of streptomycin in meat with a concentration from 0.06 mg/g to 1.99 mg/g (Dipeolu and Alonge, 2002).

Those potentially hazardous products contaminated with antibiotic residues when ingested can alter the gut microflora and increase emergence and selection for bacterial resistance in the gastrointestinal tract of humans (VICH, 2013). Moreover, when international guidelines for maximum residue limits are trying to control the limit of residues in food (WHO, 2008; Codex Alimentarius, 2018), residues are spreading through fresh water used for human and animal usage and agriculture.

Furthermore, manure originated from farms that uses antibiotics might be a pathway to help antibiotic resistance spreading from animals to humans through its usage as fertilizer in crops intended for human consumption (Kumar *et al*., 2005; Tang *et al*., 2015). However, resistant bacteria on crops intended for human consumption might be present unrelatedly to farming systems and geographical locations, and

maybe to the natural occurring and old existence of antimicrobial resistance in soil bacteria (Marti *et al*., 2013). Moreover, the occurrence of resistant bacteria on vegetables sold for human consumption was not increased by fertilizing the soil with manure but, a high level of resistant bacteria was found when vegetables were harvested with the presence of manure in soil (Marti *et al*., 2013).

After discussing the effect of antibiotic residues on consumer's microflora, bacterial resistance and its spreading to food from animal origins, we conclude that antibiotic resistance is an important worldwide animal and human health threat affecting food safety and security, animal production and economic development (FAO, 2017_a; OIE, 2015; Landers *et al*., 2012).

Existing evidences show that consumers are at risk of food-born antimicrobial resistance (Marti *et al*., 2013; Kumar *et al*., 2005; Tang *et al*., 2015; VICH, 2013; Dipeolu and Alonge, 2002; Schouten *et al*., 1997; Skov *et al*., 2007; Wilson, 2003; Warren *et al*., 2008; Ozawa *et al.*, 2002l; Noor Uddin *et al*., 2013; Zhao *et al.*, 2003; Hong *et al*., 2011) and their transmission mechanism might occur through direct contact with animals carrying the resistant pathogen or their manure, or indirectly by exposure to food contaminated from animal derived resistant bacteria (National Research Council (US), 1999). Each year, can be estimated that seven hundred thousand humans are dead because of bacterial resistant infection and an uncounted number of sick animals are not responding to antibiotic treatments (FAO, 2017_a). Furthermore, the European Centre for Disease Prevention and Control (ECDC) and the European Food Safety Authority (EFSA) have released data proving that antibiotics that are used to treat common infection between humans and animals such as campylobacteriosis and salmonellosis are nowadays less effective (EFSA, 2019_a). The European Commissioner for health and food safety "Vytenis Andriukaitis" has

rang the alarm bells, because some infections are becoming more difficult to treat than before and sometimes impossible to be treated (EFSA, 2019$_a$). Not only, but the European Commissioner is inviting all countries to support antimicrobial resistance program and insisting to work all together under the One Health Umbrella; "So before the alarm bells become a deafening siren, let's make sure that we increasingly act all together, in every country and across the public health, animal health and environment sectors under the One Health approach umbrella." (EFSA, 2019$_a$). Moreover, the rapid increase of agriculture production system has catalyzed the use of antibiotics that is expected to reach in 2030 more than double the quantity that we use nowadays (FAO, 2017$_a$).

2.4 TECHNOLOGICAL ISSUES

Long time ago, bacteria was used to ferment food for many reasons such as stability for a longer shelf life, better functionality, texture and flavor (Hill *et al*., 2017). Evidences shows that 7000 years ago, early Europeans have produced cheese (Salque *et al*., 2013), in another words, fermentation was in the base of food processing from old ages using indigenous microbial population until nowadays when technology is helping with the preselection of starter cultures with specific characteristics (Hill *et al*., 2017). Today, dairy factories are very dependent from starters that are prepared culture of microorganisms used in the production of different dairy products such as cheese, butter, yogurt, and cultured milk (EMA, 2000; Todar, 2012). Acid is produced by all dairy starter cultures including the lactic acid bacteria which are the most important one which is included in most dairy starter cultures. Veterinary residues may affect dairy industry production, by inhibiting bacterial starter cultures activity. This inhibition may lead to a slow or complete absence of acid production in fluid milk, decrease in viscosity and in obtaining the correct flavor in various products in cheese

and other milk derived products (EMA, 2000; Kosikowski and Mocquot, 1958; Fonseca *et al*., 2009; Packham *et al*., 2001; Katla *et al*., 2001). Pasteurizing milk (heating for 72 ºC) before initiating the production activities is a basic step in every dairy plant, but it is not effective in eliminating veterinary residues from milk (Tian *et al*., 2016). For instance, a study was done in Sardegna from Sarda sheep at AGRIS Sardegna Research Agency (Olmedo, Sassari, Italy) in order to evaluate thermal treatment effect of oxytetracyclin in ovine milk. Milk was spiked with half MRL (50 μg kg−1) and MRL (100 μg kg−1) level concentration, and heated until 63 ºC and directly cooled until 38ºC. Result shows that thermal treatment did not decreased oxytetracyclin concentration in all milk samples (Cabizza *et al*., 2018).

Different studies were done by Shahani, using dairy milk to evaluate the stability of chlortetracycline, oxytetracyline and penicillin G in heated milk on different temperatures and during different period of heating. Results show that heating milk spiked with chlortetracycline (0.30--0.51 μg/mL), oxytetracycline (0.32-3.22 μg/mL) and penicillin G (0.13-0.96 I.U./mL) on 62ºC during 30 minutes will reduce the percentage of residues by 16.6%, 23.6% and 8.2% respectively. When milk spiked with chlortetracycline (0.20--0.62 μg/mL), oxytetracycline (0.40-1.29 μg/mL) and penicillin G (00.25-1.07 I.U./mL) is heated for 71 ºC during 15 minutes, residues are affected by 27.6%, 35.6% and 10.1% respectively. When milk was heated for 15 minutes on a temperature of 121 ºC, chlortetracycline (0.40-0.50 μg/mL) and oxytetracyclin (0.50-0.55 μg/mL) were totally reduced but penicillin G (0.25-1.04 I.U./mL) was only reduced by 59.7% (Shahani, 1957, 1958; Shahani *et al*., 1956). Another study was evaluating streptomycin and neomycin stability when milk is heated. Milk samples were spiked with a concentration of 1 mg/ml with both molecules separately and heated for 30 minutes. When milk reached a temperature of 70ºC,

streptomycin concentration was reduced by 8.3% and neomycin by 10%. At 100 ^{o}C, streptomycin concentration was reduced by 41.7% and neomycin by 35% (Konecny, 1978). These results prove that even when contaminated milk is pasteurized, residues will remain and might pose risks to consumers. Concerning cheese production, cheese starters have different sensitivity levels for the same antibiotics. For instance, penicillin residues can cause a significant inhibition for the following bacteria on the following dosages (I. U. per ml). *Streptococcus cremoris* 0.05-0.10, *Streptococcus lactis* 0.10-0.30, *Streptococci starter* 0.10, *Streptococcus thermophilus* 0.01-0.05, *Streptococcus faecalis* 0.30, *Lactobacillus bulgaricus* 0.30-0.60, *Lactobacillus acidophilus* 0.30-0.60, *Lactobacillus casei* 0.30-0.60, *Lactobacillus lactis* 0.25-0.50, *Lactobacillus helveticus* 0.25-0.50, *Lactobacillus citrovorum* 0.05-0.10, *Propionibacteriuon shermanii*, 0.05-0.10 (table 4) (Kosikowski and Mocquot, 1958).

Table 4: Sensitivity level of microorganisms (*B.thermophilus* var. *calidolactis*) important for technological aspect to antibiotics: (Ottavio Salvadori Del Prato, 2001)

Culture	Penicillin I.U./ml	Streptomycin µg/ml	Chloramphenicol µ/ml	Chlortetracycline µ/ml	Oxytetracycline µ/ml
S. thermophilus	0.0017-0.17	0.5-5	0.05-1	0.001-0.01	0.001-0.01
S. cremoris	0.05-0.1	-	-	-	-
L. bulgaricus	0.3-0.6	-	0.3-5.0	-	-
Butter Starter	0.017-0.17	0.1-0.2	0.1-0.2	0.01-0.1	0.01-0.1
Cheese Starter	0.05-0.2	0.04	0.04	0.02-0.25	0.01
B. stearothermophilus* var. *calidolactis	0.001-0.008	0.6-1	1	0.6-1	1

Moreover, cheese making plants production system is affected as well with antibiotic residues (Ottavio Salvadori Del Prato, 2001; Charles Alais, 2000). In normal cheese production system, lactobaccillus LAB decrease cheese pH in order to prevent any microorganism multiplication and reach the fermentation level of the product (Ottavio Salvadori Del Prato, 2001). Coliforms are completely inhibited when pH reach lower levels than usually used in cheesmaking (almost 4.2). But in this case there is also the competition with LAB that controls the coliforms growth. When antibiotic residues contaminate the milk, LAB is inhibited easily (0,001 IU of penicillin is needed to inhibit LAB multiplication). The residues inhibit LAB growth and acidification, and pH remain at higher level than usual (>6), giving a chance to antibiotic unsensitive bacteria such as *E. coli*, *coliforms* and yeast to proliferates in the cheese wheel (Ottavio

Salvadori Del Prato, 2001; Charles Alais, 2000). Usually in normal process a decrease of pH at production (24 h) is expected to be around 5.0-5.2 or lower.

The heterofermentative microorganism's growth can appear within 24 to 48 hours because of early cheese blowing, usually caused by coliforms and yeasts (Ottavio Salvadori Del Prato, 2001; Charles Alais, 2000). In both cases, the heterofermentation will produce carbon dioxide gas leading to the appearance of cheese blowing and explosion (Ottavio Salvadori Del Prato, 2001; Charles Alais, 2000). Only 100-1000 UFC/g of microorganism can launch a blowing activity in the cheese wheels (Ottavio Salvadori Del Prato, 2001).

A study was done in Spain using Antibiotic-free milk that was obtained from the experimental herd of Murciano-Granadina goats of Universitat Politecnica de Valencia. 100 kg of milk were divided into 2 equal samples, one for control and the other sample was spiked with amoxicillin and benzylpenicillin (4 µg/kg), cloxacillin (30 µg/kg), erythromycin (40 µg/kg), ciprofloxacin, enrofloxacin, and oxytetracycline (100 µg/kg) at the MRL level according to the recommendations of the International Dairy Federation (ISO/IDF, 2003; Quintanilla *et al*., 2019). All standards were supplied by Sigma-Aldrich Quimica S.A. (Madrid, Spain) (Quintanilla *et al*., 2018). Results show that antibiotics affected cheese acidification by inhibiting LAB growth acidification or increasing the normal time of cheese to reach final pH level (Quintanilla *et al*., 2018). For instance, cheddar cheese made from milk containing 0.1-0.15 unit of penicillin/ml milk shows a fermented flavor and a fragile, pasty body after 3 months of ripping (Hunter, 1949). Moreover, when only 0.25-0.31% acidity are developed in 7 hours because of antibiotic residues contamination, cheese flavor is affected negatively as well as the physical composition that becomes loose and pasty (Bradfield, 1950). In addition, Camembert cheese becomes gassy when manufactured

with milk containing 0.5-1.0-unit penicillin/ml (Jacquet, 1953). Those results prove that the starter cultures activity can be strongly inhibited by the presence of those antibiotics in milk (Quintanilla *et al*., 2018; Mullan, 2003; Moghadam *et al*., 2016; Jožef and Svetozar, 2008). For a concentration below MRL, erythromycin (16 µg/L) will reduce by 50% the activity of *Streptococcus* spp. that was isolated from dairy products such as yogurt, sour cream, fermented milk, whey, cheese, and other commercial starter cultures (Katla *et al*., 2001).

Animal and human health, environment, and bacterial resistance are affected by antibiotic residues. Not only, but dairy factories also have a big technological issue when contamination of antibiotic residues occurs (Pawar *et al*., 2012). Unfortunately, antibiotic residues are not totally eliminated or destroyed by heating and cooling treatments at dairy industrial level (Cabizza *et al*., 2018; Konecny, 1978; Shahani, 1957, 1958; Shahani *et al*., 1956). Their presence even in small quantities can affect the production system by inhibiting LAB growth and stop cheese fermentation leading to the multiplication of unwanted microorganisms (Pawar *et al*., 2012; Ottavio Salvadori Del Prato, 2001; Charles Alais, 2000). Those microorganisms will affect not only the quality of the production but increases economical loses as well by obliging dairy factories to destroy all affected production and cleaning the equipment used to this production (Pawar *et al*., 2012). Moreover, dairy plants will face a disruption on the production schedule level and help in contaminating the environment while cleaning their equipment (Pawar *et al*., 2012).

2.5 LEGAL BASIS FOR FAIR FOOD MARKET

Knowing that human health depends from animal health and the environment surrounding them, care should be taken in order to prevent any risk affecting this trio

(WHO, 2017$_b$). To prevent and control any incompliancy, competent authorities in developed countries such as European Union has established different guidelines, and rules related to the prudent use of veterinary antibiotics and food safety from the farm to consumer's table.

Nowadays, the One Health Approach is being establish in order to modify and implement new programs, policies, and researches where different sectors including food safety, zoonosis control and antibiotic resistance programs work together to reach better public health results (WHO, 2017$_b$).

Humans have the right to adequate food (FAO, 2010), and by adequate FAO means that food for human consumption must be free from adverse substances, such as veterinary drug residues (FAO, 2010).

In order to protect consumers from veterinary residues, competent authorities have established regulation, guidelines and risk assessments to prevent any contamination of food from animal origin such as milk, liver, kidneys, eggs etc. (EMA, 2019; FDA, 2015). The Food and Drug Administration (FDA), the core of medicines regulations in the United States of America (USA), have set the tolerance level which is the maximum permitted concentrations for veterinary drug residues (FDA, 2018). On the other hand, the European Union (EU) has founded the European Medicine Agency (EMA) which publishes maximum residue limits (MRLs) that was established and set by the Committee for Medicinal Products for Veterinary Use (CVMP) (EMA, 2015). Moreover, the Joint Food and Agricultural Organization/World Health Organization Expert Committee on Food Additives, (JECFA) recommends as well MRLs and work as independent risk assessment bodies (FAO/JECFA, 2019). JEFCA also advises the Codex Alimentarius (CAC), playing a role of a risk manager, and

decides whether or not to establish international standards for maximum residue limits of veterinary drugs (Codex Alimentarius, 2018).

Veterinary residues are traces of drugs administered to food producing animals for therapeutic, prophylactic, diagnostic purposes, modification of physiological functions or behavior and remains in the animal tissues, such as meat or milk, poultry, fish or honey-bees (Codex Alimentarius, 2015; FAO, 2003). Any product from administered drugs and their metabolites that remain in any edible portion of the animal tissue production, are called residues (Codex Alimentarius, 2015; FAO, 2003). Those residues might be harmful to consumers and assessed based on the drug used (safe or banned) and the maximum residue limits (MRL), that is the maximum concentration of residues resulting from veterinary drug use and forced by the Codex Alimentarius Commission to be legally recognized as acceptable in or on food (Codex Alimentarius, 2015; FAO, 2003). This MRL specify in the first part the maximum limit of every veterinary drug in any animal tissue or food from animal production with the acceptable daily intake (Codex Alimentarius, 2018). Moreover, it contains banned antibiotics that are a source of risk for consumers listed in the second part of the "Maximum Residue Limits (MRLs) And Risk Management Recommendations (RMRs) For Residues of Veterinary Drugs in Foods", and listed under the title of "Risk Management Recommendations (RMR) for Residues of Veterinary Drugs" (Codex Alimentarius, 2018). In the RMR section carbadox, chloramphenicol, furazolidone, nitrofural, olaquindox and stilbenes are antibiotics and growth promoters that are banned in Europe (Codex Alimentarius, 2018). Furthermore, a clarification concerning the main reason of forbidding those veterinary drugs is explained as well (Codex Alimentarius, 2018). Until nowadays, no safe level of chloramphenicol residues or metabolites in food were found to be safe for consumers, this is why, Codex

Alimentarius have banned chloramphenicol usage in food producing animals by driving competent authorities to control its usage in food producing animals (Codex Alimentarius, 2014). Moreover, FDA USA has prohibited chloramphenicol use in food producing animals as well as extra label use (FDA, 2018). Likewise, in the European Union, chloramphenicol was listed previously in the Council Regulation No. 2377/90 (European Union, 1990) and recently revised and listed as one of the 10 prohibited substances in Table 2 of EU documents 470/2009 and 37/2010 (European Union, 2010). Before 2005, zero tolerance policy was forcing EU and USA to reject all imported food that contains chloramphenicol residues that caused an international trade impact (Love *et al*., 2011; Tran *et al*., 2012; Wongtavatchai *et al*., 2004). In order to prevent international trading problems, European Commission established a decision that set a Minimum Required Performance Limit (MRPL) or Reference Point for Action (RPA) for chloramphenicol at 0.3 µg/kg, in which they declare that all food contaminated with chloramphenicol residues equal or above RPA will be considered as non-compliant and is not allowed to be sold in the European countries (European Union, 2005_b). Otherwise, food that contains chloramphenicol residue below the RPA will be investigated by the Competent Authorities to identify the source of contamination but there is no problem to be marketed (European Union, 2005_b). Moreover, EFSA consider all food contaminated with chloramphenicol residue below RPA are unlikely to cause aplastic anemia, reproductive or hepatotoxic effects (EFSA, 2014). Furthermore, the European Union states that setting an RPA should not be a reason to support illegal and imprudent drug use for prohibited antibiotics such as chloramphenicol (European Union, 2009).

Nitrofurans is another antibiotic that is recognized as toxic (FAO, 2014). It is used in humans as a drug of choice to treat urinary infections (FAO, 2014). When food

is contaminated with nitrofurans residues, it is considered unfit for human consumption (Codex Alimentarius, 2018; FAO, 2014). This antibiotic is banned by the European Union for food producing animal, classified in the annex IV of the Council Regulation 2377/90 of the European Council in the list of pharmacologically active substances of which no maximum residue limits can be set (Council Regulation, 1990; Codex Alimentarius, 2018). Furthermore, EU has set a minimum required performance limit (MRPL) or a reference point for action (RPA) that should not exceed 1 μg/kg residues of nitrofurans to check imported products of animal origin from third countries (EFSA, 2015_a; European Union, 2005_b). Moreover, FDA prohibited nitrofurazone and furazolidone (antibiotic) use as well in 2002 (FAO, 2014). Australia banned nitrofurans in late 1992 (FAO, 2014). In Japan, no MRLs are available for nitrofurans, but no residues are allowed to be present in food (FAO, 2014). Not only the Ministry of Health in Thailand declared in 2001 a proclamation (No. 231 MRL of veterinary drug in food) in which no MRL are available for nitrofurans but also the Ministry of Agriculture and Cooperatives had previously (1999) blocked importation and use of furazolidone and nitrofurazone in feed and extended the banned list to reach nitrofurans in 2002 (FAO, 2014). As for furazolidone and nitrofurazone, they were removed from the veterinary drug formulations list in 2002 (FAO, 2014).

The European Union have established directive 96/23/EC in 1996, that aims to detect imprudent usage of veterinary drugs in animal production as well as banned substances in food producing animals (European Union, 1996). Moreover, this directive forces European member states to control and monitor groups of residues listed in annex I of this directive. Substance groups are classified in two main categories, Group A and B.

Group A is subdivided into 6 subgroups (A1 to A6), and lists unauthorized substances and substances with anabolic effect that are forbidden to be used in food producing animals in European Union and Group B lists veterinary drugs and contaminants that can be used in food producing animals in the European Union states (European Union, 1996).

Moreover, the Council Directive 96/23/EC, obliges Member States in the EU to prepare a national residue monitoring plan that complies with the sampling rules in Annex IV of the Directive, for groups of substances listed in Annex I. In addition, details concerning sampling such as frequencies and level of sampling and groups of substances to be controlled for each product are established as well in Directive 96/23 of the European Council. Each member states residue monitoring plan results, should be reported to the Commission yearly at a maximum date of 31 of March. Those monitoring plan results show all non-compliant samples in food from animal origins checked in the member state and should be set out as recommended in the repealed Council Regulation 2377/90/EEC replaced by Regulations EC/470/09 and 37/2010 (European Union, 1996). Moreover, the Department of Agriculture, Food and Marine (DAFM) manage a National Residues Monitoring plan that is intended to protect consumers from illegal residues, and therefore, samples are usually taken according with risk assessments based on standards which are built to target products or animals that have higher risk of illegal residues contamination (European Union, 1996).

Concerning hygiene on foodstuffs, the European Parliament and the Council of the European Union, have established the regulation No 852/2004, in order to ensure a healthy food for consumers (European Union, 2004$_c$). In the annex I, the part A explains the general hygiene provisions for primary production and associated operations The second point of part A, titled “Hygiene Provisions” (3;a), force food

business operators to obey specific Community and national legislative provisions that are responsible of controlling hazards in primary production systems such as contamination arising from veterinary medicinal products and others. In part (4;j) of this regulation, the European Parliament and the Council of the European Union, oblige food business operators producing primary products of animal origins to use feed additives and veterinary medicinal product prudently as required by the relevant legislation (European Union, 2004_c). Moreover, in this Regulation, ANNEX I, III. "Record-keeping", points 8(b) and 10, provided that food business operators rearing animals or producing primary products of animal origins shall keep records on veterinary medicinal products or other treatments administered to the animals, dates of administration and withdrawal periods and can be assisted by veterinarians or other competent person (European Union, 2004_c). In Annex I, part B of this regulation, voluntary guides to good hygiene practice could be developed by FBOs or their organizations and approved by the competent authorities. The guides should include appropriate information on hazards that may arise in primary production, associated operations and actions to control hazards, including relevant measures set out in Community and national legislation or national and Community such as prudent use of veterinary medicine and feed additives and their traceability (European Union, 2004_c).

Another regulation established by the European Parliament and the Council of the European Union lay down specific hygiene rules related to foodstuffs (European Union, 2004_d). In this regulation, chapter II list the food business operator's obligations, and in article 11 titled "Specific decisions", decision number 11, refers to Directive 96/23/EC forcing state members to fix a maximum permitted value for the combined total of residues of antibiotic substances in raw milk. In Annex II, Section

III titled “Food Chain Information” provided that slaughterhouses should not accept any animal without documentation concerning veterinary medicinal products administered to the animals within a relevant period and with a withdrawal period greater than zero, together with their dates of administration and withdrawal periods in order to prevent any residues in food produced by this animal. When the slaughterhouses are inside the farms, regular veterinary checks are a must. Moreover, the European Parliament and the Council of the European Union force laboratory analysis activities to assume the food safety of this animal (European Union, 2004_d). In annex III section IX, Chapter I of this Regulation, the European Parliament and the Council of the European Union have laid down requirements concerning milk and dairy products that should be complied by Food business operators producing or, as appropriate, raw milk collectors. Part B of this chapter is related to hygiene on milk producing holdings, in which they explain in the first part, that milk must be carried out hygienically ensuring in particular that animals undergoing medical treatment that is likely to transfer veterinary residues to the milk are identified and their milk is not collected before the end of the withdrawal period and is forbidden to be used for human consumption (European Union, 2004_d).

Moreover, in chapter III titled “Criteria for Raw Milk” point 4, the European Parliament and the Council of the European Union declare that based on Directive 96/23/EC, food business operators must check that raw milk is free of the banned veterinary residues. In case of allowed veterinary residues are present, they should not exceed the maximum residue level accepted by the European Union (European Union, 2009; Codex Alimentarius, 2018) or the combined total of residues of antibiotic substances should not exceed any maximum permitted value (European Union, 2004_d). When raw milk is not complying to the Regulation 853/2004, food business operators

must inform the competent authority and take measures to correct the situation (European Union, 2004$_d$).

2.5.1 Exporting food from animal origins from third countries to EU

In order to prevent European market contamination by food from animal origins, exported from third countries or developing countries to EU, that might be contaminated with veterinary residues, the European Parliament and the Council of the European Union have established a directive No 97/78/EC on 18 December 1997 laying down the principles governing the organization of veterinary checks on products entering the Community from third countries (European Union, 1997$_a$) and the Regulation of the European Council No 882/2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules (European Union, 2004$_a$).

All food from animal origins listed in European Council Directive's 89/662 and 90/425, originated from third countries introduced into any of the European territories listed in Annex I of the Council Directive 97/78, shall be checked by the official veterinarians at the borders of the European countries according to the Directive 97/78 of the European Council. The fifteen European territories are listed in Annex I of the European Directive 97/78/EC (European Union, 1997$_a$).

Moreover, competent authorities should be checking regularly for veterinary residues in food from animal origins exported from third countries to EU based on risk assessment with the appropriate frequency (European Union, 2004$_a$). On the other hand, official control should approve on food chain stages starting from the farm to table covering all stages with import and export phases. Furthermore, the European Union has established a framework for designation of European Reference Laboratories listed under the title of "COMMUNITY REFERENCE

LABORATORIES" in annex VII of the REGULATION (EC) No 882/2004. Those accredited laboratories are references for the European Union member states to check for any non-conformities in all food from animal origins exported from third countries (European Union, 2004_a). The governing principles of the veterinary organization that checks on products entering the Community from third countries, aims to protect the health of citizens and animals inside the European Union (European Union, 1997_a). Documentary checks, related to veterinary certificates, identity check of the products and physical checks (such as laboratory testing for veterinary residues) are the three basic checks to be performed on exported food from third countries at the European borders (European Union, 1997_a). All products that are not compliant with the European regulation are destroyed or returned (European Union, 2004_a; European Union, 1997_a). When the European country detects any non-conformity in any imported product from a third country, the member state should immediately inform the country of origin about the findings. In the event that non-compliance is detected again from the same country of product origin, the member stated of the destination country should inform the European Commission to take action and the other European Countries as well (European Union, 1997_a). Concerning banned antibiotics in food from animal origins, the European Union enforce checking all food from animal origins imported from third countries to the European Union territories, based on the minimum required performance limit (MRPL) or a reference point for action (RPA), before entering EU borders, regarding to the Commission Decision 2005/34/EC that force the testing procedure of the minimum required performance limit or reference point for action (European Union, 2005_b). All food contaminated with residues at or above the RPA of μg/kg of Chloramphenicol is considered non-compliant and removed from the food market chain (destruction, re-dispatch, recall). Repetitive

findings below the RPA, indicating a recurrent pattern of banned antibiotic usage, will trigger specific actions directed towards the third countries of origin (European Union, 2005_b). The same procedure is applicable between two European countries when food from animal origins is the trading product (European Union, 1996). Those regulation and decisions are confirmed by Regulation (EC) No 470/2009 (European Union, 2009).

If a third country is willing to export any animal or animal product to any European Member States, it is imperative that they have a residue monitoring plan that is in accordance with the Council Directive 96/23 of the European Council, for all food from animal origins. Moreover, this 3rd country must appear on the list of countries approved for residue monitoring plan (European Union, 1996).

Chapter 3: Antibiotic and antibiotic residues management strategy

3.1 COMPETENT AUTHORITIES ROLE

Antibiotics are used worldwide in food producing animals (FAO, 2019$_a$). Their residues may reach consumers in case of imprudent veterinary antibiotics usage occurs. The National food control system is based on many principles in order to reach a safety level for consumers. This plan aims to protect consumer's health and guarantee fair food trade activities (Codex Alimentarius, 2013).

The 1st principle of this plan is to protect consumers, especially when different interests are in conflict.

The 2nd principle is to assure a protection wave that covers the whole food chain from farm to table.

The 3rd principle is about transparency and respecting legal needs with confidentiality. This transparency is necessary to all food chain handlers and should be achieved through clear documentation and communication.

The 4th principle defines roles and responsibilities of all parties that can affect food from animal origins production explaining that food safety is the responsibility of business operators and commercial entities that are involved in the production, processing and marketing of food (Codex Alimentarius, 2009; 2013). As for the Competent Authorities, their role is to regulate veterinary drugs use, verify that drug handler's activities are appropriate to the European guidelines and that drug distribution and food industries practices are under effective measures in order to

guarantee an efficient safety level for consumers and increase the safety of trading between countries under Codex Alimentarius objectives. Any competent authority that is responsible for ensuring healthy food for it citizens, must ascertain that it have enough knowledge and control over food safety programs as well as veterinary drugs that are sold and used in its market for the food production activities (Codex Alimentarius, 2009).

Knowing that all European member states have the same requirements of Codex Alimentarius, residue management criteria should be established by the competent authorities of each member states and maintain the same safety level to be accepted by other European member states in order to fulfill food quality, safety and trading needs.

Each production system and region have different risk profiles, this is why the competent authority in each member states such as Ministry of health and Ministry of Agriculture, are free to build their control system by respecting Codex Alimentarius norms (Codex Alimentarius, 2009).

The official approval systems are based on two main requirements that are:

a) Evaluation of veterinary drug residues on human safety based on risk analysis and establishing a maximum residue limits when needed.

b) Taking into consideration producers requirements to decrease the likeliness of banned and unapproved veterinary drugs use (Codex Alimentarius, 2009).

Based on those requirements, the national authorities should approve on all veterinary drugs before selling them for use to guarantee a safe level of drug usage in the European territories (European Union, 2001). Moreover, they must establish a simple registration protocol for homeopathic veterinary drugs. They have to make sure that drug factories and distributers are authorized, and working on an expert level by

respecting the national authorities law. Not only but national authorities are responsible of encouraging reporting systems on any adverse reactions on veterinary drugs. In order to correct any incompliancy, national authorities are in charge of regular inspections and laboratory tests to ensure that drug factories are working within the national legislation. When inspections results are respected, a "Certificate of Good Manufacturing Practice" should be granted to the drug manufacturer by the national authorities and the inspection results should access the European database within 90 days maximum. The authorization of any drug that is considered dangerous or without any beneficial effect will be suspended or revoked. Only in case of a serious national outbreak infection, national authorities can use this drug even if it is not yet authorized after the European Commission approval (European Union, 2001). The European Council Directive 2001/82/EC will be repealed and replaced by the European Council Regulation (EU) 2019/6 as of 28 January 2022.

3.2 REGISTRATION OF DRUG SYSTEM

In 1993, the European Union has published the regulation No 2309/93 in which they lay down Community procedures for human and veterinary medicine authorization and supervision (European Union, 1993). Moreover, they established a European agency for the Evaluation of medicinal Products that have to publish a general report on the operation and procedures within six years post-forcing the regulation (European Union, 1993). The report of the European agency for the Evaluation of medicinal Products has proved that it needs to amend certain governmental role and that improving the authorization procedures for placing of medicinal products on the market is essential. In addition, the European agency for the Evaluation of medicinal Products is renamed with European Medicines Agency and referred to "Agency" (European Union, 2004_b).

Three different routes are available for veterinary drug authorization that are the centralized procedure, decentralized procedure and mutual-recognition (EMA, 2016$_a$).

Almost all drugs authorized in the European Union are authorized by national competent authorities (NCAs) in the Member States using decentralized procedure and mutual-recognition in order to use those drugs in several member states but the centralized procedure is used to authorize novel medicines including drugs for rare diseases (EMA, 2016$_a$).

- The centralized procedure starts by submitting a single authorization application to EMA by pharmaceutical companies. After that, Committee for Medicinal Products for Veterinary Use (CVMP) evaluates the application and shares her recommendation with the European Commission in order to grant or not the marketing authorization. When the drug is granted, the centralized marketing authorization is valid in all European member states.

- The decentralized procedure is applied at the same time for a drug that is not complying with the centralized drug requirements, not yet authorized in any European member states and the pharmaceutical company needs to authorize the drugs in more than one European member states.

- The mutual-recognition procedure is used to approve a single European member state authorization of a drug in other European countries. Knowing that the Codex Alimentarius and the European Medicinal Agency are unique

for all European Countries, this procedure allows European member states to trust each other's national scientific evaluations (EMA, 2016$_a$).

European citizens have the right to understand how decisions and regulations are reached, this is why a European Public Assessment Report (EPAR) for any veterinary drug authorization is published in details with an assessment by (EMA) even if the drug has been approved or not for authorization (EMA, 2016$_a$).

Comparing United States Food and Drug Administration (FDA) with European Medicines Agency, a requirement by the Federal Food, Drug and Cosmetic Act (FD&C Act), novel veterinary drugs should be reviewed by FDA to guarantee safety and efficiency and acquire official marketing status before putting them into market (FDA, 2019$_b$). Pre-market review is a must for FDA to warranty animals and public health protection (FDA, 2019$_v$). This review allows the US agency to assess materials submitted by the pharmaceutical company, to assure drug safety, effectiveness, labeling and proper manufacturing (FDA, 2019$_b$). When the novel drug acquires FDA approval, it can be legally used in the market (FDA, 2019$_b$).

Depending of the drug, approval, conditional approval and indexing are the three available ways in the US FDA in order to acquire the legal marketing status.

- Approval pathway is when a "new animal drug" (The term "new animal drug" means any drug intended for use for animals other than man (FDA, 2010) has gone through the process of the New Animal Drug Application (NADA) and has received Center of Veterinary Medicine's (CVM's) stamp of approval (FDA, 2019$_b$). If the drug data meets FDA requirements, then it is considered as safe for the market and the information on the drug label are submitted by the pharmaceutical company (FDA, 2019$_b$).

- Conditional approval is a yearly pathway available for drugs designed for minor species or for minor usage in major species (FDA, 2019b). This conditionally approved drug has passed through FDA approval process but is not yet ready to meet FDA's full approval. When this drug is used based on its label, it is safe and has a "reasonable expectation of effectiveness". This pathway can be renewed annually for 5 years if the pharmaceutical company asks FDA and the drug quality meets FDA requirements. During those 5 years, the conditionally approved drug can be sold legally in the market while collecting the remaining effectiveness data. After this period, the pharmaceutical company can ask FDA for a full approval attached with the collected effectiveness data. If the drug data meets FDA's requirements, the drug will be totally approved (FDA, 2019b).

- When a drug is listed on "FDA's Index of Legally Marketed Unapproved New Animal Drugs for Minor Species", it is called "indexed animal drug". This drug can be legally used for specific use in some minor species such as non-food-producing minor species and early non-food life stage of a food-producing minor species. The indexing process depends from qualified experts outside FDA that will review the drug safety and effectiveness in the specific minor species animals. All experts together should approve on the drug's benefits outweighing the risks to the treated animal, therefore FDA will add the drug to the index (FDA, 2019b).

Veterinary drugs approval by the member states should be controlled as well with some restrictions forced by national regulations in order to decrease potential risks imposed by their use. Restrictions are enforced on the drug formulation, indication of use, route of administration, criteria of the drug and drug withdrawal

period (Codex Alimentarius, 2009). When a veterinary drug is approved by the member state's official control, it should be registered in the European Union national registration system. Moreover, the member state of the European Union should build educational programs or force the manufacturing company or the pharmaceutical agent of the approved drug, to give correct information on the new drug by training veterinarians or drug handlers on the prudent use of this approved drug to use it without affecting consumer's health. At the end, competent authorities are responsible of establishing and maintaining legal requirements and ensuring an effective national food control system operation (Codex Alimentarius, 2009).

In order to maintain and ensure a safety food for consumers, the European parliament and the council of the European Union force the competent authorities of the European Member States in regulation No 882/2004, to work in collaboration with official laboratories to analyze samples collected during official controls and to ensure the verification of compliance with feed, food, animal health and animal welfare rules (European Union, 2004_a).

Laboratories working for national and member states authorities should be accredited and operating based on an international standards working with validated methods with particular standardized equipment in order to detect any non-compliancy referring to the competent authority maximum residue limit. Their results should be uniform and private, tested with objectivity and training is necessary for all laboratory workers (European Union, 2004_a).

The accreditation of the laboratories should be according to the European standards:

"- EN ISO/IEC 17025 on "General requirements for the competence of testing and calibration laboratories";

- EN 45002 on "General criteria for the assessment of testing laboratories";

- EN 45003 on "Calibration and testing laboratory accreditation System-General requirements for operation and recognition" (European Union, 2004_a).

In the annex VII, article 32 of the regulation No 882/2004, the European parliament and the council of the European Union laid down the community reference laboratories responsibilities to the national reference laboratories, such as sharing the reference method used with details of the analytical methods, organizing comparative testing and follow-up with international protocols, informing about new analytical methods depending from their area of competence and providing technical and scientific assistance to the member state commission. Moreover, they should conduct training courses in collaboration with national reference laboratories to 3rd countries laboratories and collaborate with those responsible of feed and food analysis (European Union, 2004_a).

Furthermore, Community reference laboratories must cover European requirements such as, having a qualified competent trained staff, equipped with calibrated machines and accredited products to fulfill the requirements of needed tests, proper administrative infrastructure, staff respecting confidentiality, sufficient knowledge to European International Standards and practices with a trained personnel for laboratories emergency cases (European Union, 2004_a).

In addition, the European parliament and the council of the European Union has forced in the regulation No 882/2004 annex VII article 33, the European member states to refer one or more national reference laboratories for each community reference laboratory. Moreover, the national reference laboratory can be a reference for different member states and the member states community reference laboratory might be

referred to a national laboratory in another member state of any European Free Trade Association (EFTA) member (European Union, 2004_a).

Furthermore, national laboratories are obliged to collaborate and check the results shared from the community reference laboratories with a comparative test between different community reference laboratories. Not only they have a primary role in ensuring the dissemination of information supplied by the community reference laboratories to the competent authorities and national laboratories, but also, they have to fulfill technical and scientific trainings to the competent authorities in order to implement a coordinated control plan. When the member state has multiple national reference laboratories for a community of reference laboratories, they have to be sure that these laboratories are collaborating between each other and with the other national laboratories as well as the community reference laboratory (European Union, 2004_a).

Moreover, the European Commission and the Council of the European Union has laid down Decision 2002/657/EC in which they stated analytical methods rules that should be used by the accredited laboratories in the residue monitoring plan while testing official collected samples and specifying standards to interpret the analytical results of official control laboratories for such samples (European Union, 2002_b).

To ensure the safety of veterinary usage, the European parliament and the council of the European Union has established the directive 2001/82/EC of the European Council related to veterinary medicinal products (European Union, 2001). This directive set out the European rules on authorization, manufacturing, supervision, sale, distribution and use of veterinary medicinal products (European Union, 2001).

The directive 2001/82/EC of the European Council will be repealed and replaced by the European Union Regulation (EU) 2019/6 as of 28 January 2022 (EUR-Lex, 2019).

As for the European Union regulation (EU) 2019/6 on veterinary medicinal products it aims to modernize legislation, stimulate innovation and increase the availability of veterinary medicinal products and fortify the European's campaign towards antimicrobial resistance (European Union, 2019$_a$). The regulation (EC) 2019/6 is part of an improvement package in animal and human health that includes as well the regulation (EU) 2019/4 that lays down the rules of manufacturing, marketing and usage of medicated feed as well as the regulation (EU) 2019/5 on authorization and supervision process of medicinal products for humans and animals and establishing a European Medicines Agency (European Union, 2019$_{a,b,c}$).

By the European Union Regulation (EC) 2019/6, veterinary medicinal products are defined as "any substance intended for animals" and used; to treat and prevent a disease, change animal's physiological functions by affecting the immune system or metabolism, medical diagnosis and euthanasia (European Union, 2019$_a$).

Concerning legislative modernization, the European Union Regulation (EC) 2019/6, harmonizes labelling requirements and embraces an easier system for exception's decisions. As for pharmacovigilance, a risk based approach is in the framework in order to control the effectiveness of the system (European Union, 2019$_a$). Moreover, this regulation forces all marketing authorization to be only given by the European Commission or the competent authority and clinical trials should not be done without official approval in order to protect laboratory animals in scientific experiments. Moreover, official authorization is necessary for any participation at any manufacture of veterinary medicinal products stage or for veterinary drug imports (European Union, 2019$_a$).

Furthermore, the European parliament and the council of the European Union has found that stimulating innovation and completion can affect positively the

availability of veterinary medicinal products, this is why the European Union Regulation (EC) 2019/6 launches a simple assessment method with a data protection period that can be extended for up to 18 years in order to stimulate the development of novel veterinary antibiotics and veterinary medicinal products for rare diseases as well as for bees and pets (European Union, 2019$_a$).

In addition, this regulation describes new rules for novel therapies methods and organic veterinary drugs. Furthermore, in order to promote novel veterinary medicinal products new rules are established to enlarge the veterinary drugs range that can be authorized with the centralized procedure (European Union, 2019$_a$).

Antimicrobial resistance is covering a big part of nowadays challenges, this is why the European Union regulation 6 of 2019 continues to support the European Union war against antimicrobial resistance by banning the preventive use of antibiotics via medicated feed in groups of animals and restricting metaphylaxis antibiotic use.

The European Union Regulation (EC) 2019/6 also reinforces the restriction on growth promoters and yield increasers. Moreover, some antibiotics will possibly be reserved for human use only and the European Union countries are obliged to collect data related to the sale and use of veterinary antibiotics. In addition, the European Union Regulation (EC) 2019/6 notifies the non-European countries to respect their rules related to banned veterinary drugs and those reserved for the human use in the European Union, in order to increase the protection level of the consumers and European citizens against antimicrobial resistance spreading through live animals and imported animal products (European Union, 2019$_a$).

In the European Union Regulation (EC) 2019/6 Chapter VII titled “Supply and Use” the European parliament and the council of the European Union oblige the wholesale distributers to earn the European accreditation in order to have the right of

working with veterinary drugs. Moreover, they have to buy veterinary drugs from pharmaceutical companies or industries only, comply with the good distribution practices and permit only for a qualified competent person to carry out retail activities in a member state (European Union, 2019a). The wholesale distributor should keep at least the following detailed records about each transaction; "date of the transaction; name of the veterinary medicinal product including, as appropriate, pharmaceutical form and strength; batch number; expiry date of the veterinary medicinal product; quantity received or supplied, stating pack size and number of packs; name or company name and permanent address or registered place of business of the supplier in the event of purchase or of the recipient in the event of sale". Furthermore, the wholesale distributor has to complete a detailed audit and records should be achieved for competent authority's inspection (European Union, 2019a).

In the chapter VII of the European Union Regulation (EC) 2019/6, article 102 forces the parallel trade rules by obliging the wholesale distributer to make sure that veterinary drugs from the member state (exporter) and sold in another member state (importer) are authorized and identical in pharmacokinetics and pharmacodynamics terms, in the destination member state and manufactured by the same authorized manufacturer (European Union, 2019a).

The veterinary drugs retailers should be authorized and buy veterinary drugs from the wholesale distributers only. Moreover, records should be kept for each veterinary prescription including; "date of the transaction; name of the veterinary medicinal product including, as appropriate, pharmaceutical form and strength; batch number; quantity received or supplied; name or company name and permanent address or registered place of business of the supplier in the event of purchase, or of the recipient in the event of sale; name and contact details of the prescribing veterinarian

and, where appropriate, a copy of the veterinary prescription; marketing authorization number" and a detailed audit should be done for every retail at least once a year and results should be archived for the competent authorities controls (European Union, 2019$_a$).

As for the antibiotic veterinary prescriptions for metaphylaxis purposes, they can only be issued when an infectious disease is diagnosed by a veterinarian justifying the antibiotic usage purpose not only for such cases but also for any antibiotic prescription. Moreover, a veterinary prescription is only issued after a veterinary examination of the animals. Any veterinary prescription should answer the following details; "identification of the animal or groups of animals to be treated; full name and contact details of the animal owner or keeper; issue date; full name and contact details of the veterinarian including, if available, the professional number; signature or an equivalent electronic form of identification of the veterinarian; name of the prescribed medicinal product, including its active substances; pharmaceutical form and strength; quantity prescribed, or the number of packs, including pack size; dosage regimen; for food-producing animal species, withdrawal period even if such period is zero; any warnings necessary to ensure the proper use including, where relevant, to ensure prudent use of antimicrobials; if a medicinal product is prescribed for metaphylaxis, prophylaxis or a banned/prohibited antibiotic usage" (European Union, 2019$_a$).

The prescribed drug quantity should be limited to the treatment requirements and the antibiotics prescribed for metaphylaxis or prophylaxis purposes should be limited to cover the period of risk. As well all prescribed drugs should be supplied by respecting the competent authorities law. Moreover, the prescription is valid for 5 days from the issue date and veterinarians should keep records for every issued prescription (European Union, 2019$_a$).

According to the applicable national law, only a veterinary drug can be used without prescription if prudently administered directly by a veterinarian, but records are necessary in all veterinary drug usage cases (European Union, 2019$_a$).

In the chapter VII of the European Union regulation (EC) 2019/6, article 107 of the European Union Regulation (EC) 2019/6, the European parliament and the council of the European Union impose a law on veterinary antibiotic use, explaining that it is forbidden to use them often to compensate lack of hygiene and care, poor animal husbandry and farm management, and not even for growth promoters and increasing the yields. Antibiotics can be used exceptionally as a prophylactic and metaphylactic treatment in an infectious disease case to prevent the dissemination of the infection, for a limited period of time and when no other treatments are available. When restricted antibiotics are used for specific cases, the member states should be informed to inform in their turn the national authorities, and risks concerning animals, public health and environment resulting from their usage should be taken into consideration (European Union, 2019$_a$).

Not only veterinarians but also owners and keepers of food-producing animals should keep veterinary antibiotic treatment records and a copy of the veterinarian prescription. Records should include; “date of the first administration of the medicinal product to the animals; name of the medicinal product; quantity of the medicinal product administered; name or company name and permanent address or registered place of business of the supplier; evidence of acquisition of the medicinal products they use; identification of the animal or group of animals treated; name and contact details of the prescribing veterinarian, if applicable; withdrawal period even if such period is zero and the duration of treatment”. Those records should be archived for at least 5 years to fulfill official authorities control needs (European Union, 2019$_a$).

In the chapter VII of the European Union Regulation (EC) 2019/6, article115, the European parliament and the council of the European Union oblige the veterinarians to respect the withdrawal period of the used antibiotic. For instance, the withdrawal period for milk from milk-producing animals intended for human consumption should not be less than 1.5 times the withdrawal period defined by the product manufacturer, 7 days in case of usage of a drug that is not authorized for animal producer milk for consuming and one day if the drug has zero withdrawal period (European Union, 2019$_a$).

In order to be certain that veterinary drugs are manufactured, sold, and used prudently, the European parliament and the council of the European Union has established the article 123 in the Regulation (EU) 2019/6 obliging the competent authorities to carry out a control system on the following parts; "manufacturers and importers of veterinary medicinal products and active substances; distributors of active substances; marketing authorization holders; holders of a wholesale distribution authorization; retailers; owners and keepers of food-producing animals; veterinarians; holders of a registration for homeopathic veterinary medicinal products; holders of prohibited veterinary medicinal products and any other persons having obligations under the European Union Regulation (EC) 2019/6" (European Union, 2019$_a$).

The European control system activities are regularly performed, based on a risk assessment to make sure that all parts that are in relation with veterinary drug usage are complying with the European Union Regulation (EC) 2019/6. This risk based controls system should be established in relation with the intrinsic risks associated with the activities of the people that should be controlled, the history and results of old control activities referred to that person, any non-compliancy hint and the effect of non-compliancy on consumers, animal and environment (European Union, 2019$_a$).

Moreover, another member state can request a control activity to be performed on some personel in other member states. Any control should be performed by the competent authority's representatives, and inspections can be done as part of this control as well, and they might be done unannounced with a secret scheduled calendar. "The official control representative can inspect the premises, equipment, means of transport, records, documents and systems, related to the objective of the inspection. Moreover, inspector can take samples with a view to submitting them for an independent analysis by an Official Medicines Control Laboratory or by a laboratory designated for that purpose by a Member State, as well as any document that might serve as evidence. All records for each inspection should be kept by the official control representative member, and used to present an official report for the competent authorities within a limited period of time set by the latter, especially for any non-compliancy detected during inspections.

Moreover, the official control should arrange the controlling procedures in order to guarantee that inspectors are fulfilling their tasks without any conflict of interest (European Union, 2019_a).

3.3 MAXIMUM RESIDUE LIMITS NORMS

Residue left from administered veterinary drugs to food producing animals might be harmful to consumer's health when they ingest food from animal origins. Those residues should be assessed scientifically based on the European Union Regulation (EC) No 470/2009 (European Union, 2019_a). In order to prevent any food contamination, maximum residue limit is established by the European Medicine Agency Committee for Medicinal Products for Veterinary Use (EMA, CVMP) (European Union, 2015) the Joint Food and Agricultural Organization/World Health

Organization Expert Committee on Food Additives, (FAO/ JECFA, 2019), Codex Alimentarius (CAC) (Codex Alimentarius, 2018) and used by the competent authority to verify the compliancy of any collected sample during a controlling procedure. European Union obliges any food from animal origins factory to verify the compliancy of his products before marketing them (EMA, 2019). The maximum residue limit (MRL) is recommended by the European Medicines Agency (EMA) Committee for Medicinal Products for Veterinary Use (CVMP), and when adopted by the European Union, they will become a reference for food safety standards (EMA, 2019).

The European Union Regulation (EC) No 470/2009, set out rules for establishing veterinary maximum residue limits for food from animal origins. Moreover, this regulation set out the basis for different regulations such as the European Union Regulation (EU) No 37/2010 that classifies the pharmacological active drugs concerning maximum residue limits in food from animal origins (European Union, 2009; 2010). In the table 1 of the annex, veterinary medicinal products allowed for food producing animals are listed with their maximum residue limits target species and tissues (European Union, 2010). In contrary, veterinary drugs listed in table 2 of the annex are banned for food producing animal usage and form a hazard to consumers when ingested in contaminated food (European Union, 2010; EMA, 2019). With every single change in the maximum residues limits, the regulation is updated (EMA, 2019).

To establish a maximum residue limit, there is an application to be completed and explained in the European Union Regulation (EU) 2017/12 that works in accordance with the European Union Regulation (EC) No 470/2009 of the European Parliament and of the Council (European Union, 2017$_a$). The European Union Commission Regulation (EU) 2017/880 provides "rules on the maximum residue limit use, established for pharmacologically active drugs in a specific foodstuff for another

foodstuff derives from the same species and a maximum residue limit established for a pharmacologically active substance in one or more species for other species, in accordance with European Union Regulation (EC) No 470/2009 of the European Parliament and of the Council" (European Union, 2017$_c$). As for the control purposes for foodstuffs derived from animals which have been treated in the European Union Directive 2001/82/EC Article 11, detailed in the previous part, the European Union Regulation (EC) No 470/2009 provides rules on the maximum residue limit to be considered for control purposes (so called 'cascade MRLs'). As for the scientific risk assessment and the establishment of risk management recommendations relevant to maximum residue limit application, the European Union Regulation (EU) 2018/782 lays down the methodology that should be used (European Union, 2018).

3.4 NATIONAL PLAN TO CONTROL ANTIBIOTIC RESIDUES IN FOOD

The national European authorities have established a plan in order to control veterinary antibiotic residues. This plan is somehow the basic design of all European member states antibiotic residue control plans. Four official papers were established by the European parliament and the council of the European Union covering all information needed to establish a control plan, with sample collection norms, laboratory criteria and validation techniques.

The European Union Council Directive 96/23/EC lays down the basis of establishing a residue monitoring plans, sampling frequency and range of substances listed in annex I to be tested (European Union, 1996). Those substances are divided into two groups, growth promoters and unauthorized substances in the first part and veterinary drugs and contaminants. This directive is repealed and replaced by the European Union Regulation 2017/625 with effect from 14 December 2019.

The national monitoring plan allocate the European member states to control antibiotic residues by forcing them to assign to a central public department the duty of establishing a veterinary drug residue monitoring plan in order to detect antibiotic residues in live animal, their excrement, tissue and animal products, animal feed, and drinking water (European Union, 1996). In its turn, the public department should organize the central and regional unit activities that are responsible of organizing and collecting data of the monitoring plan results that will be shared with the European commission (European Union, 1996).

Moreover, the directive obliges the European countries to present to the national authorities their control plans that should be complying with the sampling levels and frequencies found in annex IV of the directive (European Union, 1996).

At least a yearly report must be send from the Commission to the European countries within the Standing Committee on Plants, Animals, Food and Feed. Not only but the Commission must share a communication on the results of action taken at regional, national or European level to the European Parliament and the Council of the European Union (European Union, 1996).

Furthermore, the directive obliges the European countries to guarantee that their regulations cover the basis of quality monitoring of the food production chain by all parties that are involved and that the auto-monitoring criteria are sharp and included within the trademarks or labels specifications (European Union, 1996).

This directive also lays down controlling activities that should be performed by the official control to guarantee the food safety measures. The European countries should perform official random checks activities to the manufacture of pharmaceutical substances with growth promoter effect. These drugs should be controlled from the industrial level until the sale points. Moreover, random checks should be done to the

animal feed throughout all its process as well as the animals and raw material from animal origins (Bovine, porcine, ovine, caprine and equine animals) and production chain (European Union, 1996).

Furthermore, when a non-compliant sample shows up, competent authorities should be informed as fast as possible of the necessary information that allows treated animal identification, farm of origin and the examination results (European Union, 1996).

When controls done in a European country shows a necessity for an investigation in one or different European or non-European countries, the first must inform the other European countries and the European Commission as well. When investigations prove the necessity to take corrective measures, law does not differ in which country the non-conformity was detected (European Union, 1996).

To protect the high level of food safety, a European country is allowed to inform the competent central authority about any other European country where there is a suspicion that its control system is below the European standards. The national authority will investigate, and when needed, experts are asked for their opinions in order to take the best action (European Union, 1996).

The responsible of the non-compliancy will have to accept a penalty such as "suspending or withdrawing the authorizations or official approval arrangements or imposing criminal and/or administrative penalties and, in the event of non-cooperation with the competent authority or of obstruction, excluding any possibility of European aid being received for a period of 12 months" (European Union, 1996).

Moreover, this directive lays down rules concerning imports of animals and food from animal origins from non-European countries listed in the European legislation.

Those countries are obliged to guarantee throughout a residue control plan concerning maximum residue limits and banned veterinary drugs (European Union, 1996).

The Regulation (EU) 2017/625 aims to establish common rules for the European official controls in order to guarantee that legislation concerning the agri-food chain for the protection of human health, plant health, animal health and welfare, is correctly applied and enforced (European Union, 2017_b). Moreover, this new regulation launches a synchronized and coherent approach to official controls and enforce some actions on the agri-food chain production and fortify the principle of risk-based control system. Furthermore, this regulation as the old directive, covers all the agri-food chain cycle from primary procedures to retailers and caterers, and also plant/animal breeders, growers and traders (European Union, 2017_b).

Moreover, official controls are carried out by national enforcement authorities to verify "food and feed safety throughout their whole cycles, genetically modified organisms, animal's health and welfare, organic production, labelling, imports of animals and goods from outside the European union countries" (European Union, 2017_b).

Furthermore, the new regulation initiate the risk-based control system in order to let national enforcement authorities to control the areas that show a high risks of contamination and to ensure the official controls effectiveness (European Union, 2017_b).

On the other hand, the regulation will cover animal welfare rules including farming, transportation and slaughtering, as well as the cooperation between the European countries by clarifying and strengthening rules on the collaboration and governmental assistance between European countries, ensuring information exchange between enforcement and national authorities, public prosecutors and judicial

authorities on possible non-compliant cases and building an integrated computerized management system for official controls that will be managed by the European Commission (European Union, 2017$_b$).

Moreover, to ensure transparency in the system, national authorities must publish annual reports and new calculation rules will be used to guarantee that the official control system is not exceeding the needed amount (European Union, 2017$_b$).

Each controlling protocol needs a sampling procedure, this is why the European Commission and the Council of the European Union has established the European Union Decision 97/747/EC fixing the levels and frequencies of sampling provided by the European Union Council Directive 96/23/EC for monitoring of certain substances and residues thereof in certain animal products (European Union, 1997$_b$).

In the European Union Decision 97/747/EC chapter 1 part A titled with "Bovine milk", the European commission lays down sampling requirements rules forcing the official competent authorities to be the only one responsible for taking an official sample, and samples should be traceable back to the farm of origin as well. Samples can be taken at farm level from the collection tank or at the dairy industry before discharging the bulk tank. Samples should be taken only from raw milk and the size of it is related to the analytical method used (European Union, 1997$_b$).

In the European Union Decision 97/747/EC chapter 1-part B explains the sampling level and frequency in which they announce that the annual number of samples is 1 per 15000 tons of the annual milk production with a minimum of 300 samples (European Union, 1997$_b$).

70% of the collected samples should be checked for veterinary drug residues by searching for at least 4 different compounds from at least 3 drug groups (A6) that are

in the list of pharmacologically active substances for which no maximum levels can be fixed listed in Council Regulation (EEC) No 2377/90 of 26 June 1990, Antibacterial substances, including sulphonomides and quinolones and Non-steroidal anti-inflammatory drugs (NSAIDs) (European Union, 1997$_b$).

Fifteen percent of the collected samples should be checked for the presence of residues of "Organochlorine compounds including PcBs, Organophosphorus compounds, Chemical elements, Mycotoxins, Dyes, Others" that are designated in group B3 of the European Union Council Directive 96/23/EC (European Union, 1997$_b$).

The last 15% of the collected samples must be allocated based to the member state situation (European Union, 1997$_b$).

The European Commission has established the Decision 98/179/EC, to rule down official sampling and accreditation requirements for official laboratories (European Union, 1998$_b$). The annex of this decision lays down the responsibilities of the official inspector forcing him to be responsible of taking, registering, preparing and organizing the transport of the official control samples under appropriate conditions (European Union, 1998$_b$).

Samples should be analyzed only by approved laboratories working with the competent authorities for official residue control. Moreover, these laboratories must show their competence successfully by participating regularly in testing schemes organized by the national or community reference laboratories (European Union, 1998 $_b$).

Official sampling procedure should be unforeseen, unexpected with no specific date and time, and carried out in different intervals spread over the whole year (with

seasonal production exceptions). Furthermore, member states should guarantee to perform the procedure with the element of surprise (European Union, 1998$_b$).

Different criteria are used to select an official targeted sample on farm level such as type of the farm, breed, sex of the animal and the selection should be based on the inspector assessment that should be relied on the "indication of use of pharmacological active substances, secondary sexual characteristics, behavioral changes, the same level of development in a group of animals of different breed/categories, animals with good conformation and little body fat percentage" (European Union, 1998$_b$).

When sampling procedure should be performed at a primary processing establishment, efforts should be made to prevent big sampling number from the same producer. Moreover, different criteria play a role in the inspector assessment such as "sex, age, species, farming system, information about the producer, indication of use of pharmacological active substances, common practice with regards to the administration of particular pharmacological active substances in the respective farm production system" (European Union, 1998$_b$).

Furthermore, samples containers should maintain samples quality; prevent cross-contamination and degradation, officially sealed and suitable to maintain traceability and integrity. The sample quantity should be enough to perform complete screening and confirmatory analysis procedures by the approved laboratories. Samples should also be divided into equal sub-samples (at least 2) that each one is enough to perform the analytical procedures needed, unless the national legislation do not require the sample division or the sample cannot be divided technically. The division into sub-samples can be performed at the sampling location or in the laboratory but only by the competent personel (European Union, 1998$_b$).

After each official sampling procedure, a sampling report must be done by the inspector containing data such as the "address of the competent authorities, name of the inspector or identification code, official code number of the sample, sampling date, name and address of the owner or the person having charge of the animals or the animal products, name and address of the animal's farm of origin (when sampling on farm), registration number of the establishment-slaughterhouse number, animal or product identification, animal species, sample matrix, medication within the last four weeks before sampling (when sampling on farm), substance or substance groups for examination, particular remarks". In case of the on-farm sampling, the inspector and the farmer or his deputy should sign the original sampling report, that will be archived at the competent authorities in order to keep its access safe (European Union, 1998_b).

In order to preserve the sample quality, sample must be transported in a specific storage based on the sample quality, with defined conditions such as temperature and period from collection to delivering to the laboratory (European Union, 1998_b).

As for the laboratory tests reports, they must contain important information such as "address of the competent authorities, name of inspector of identification code, official code number of the sample, sampling date, animal species, sample matrix, substances or substance groups for examination, particular remarks". This report should be submitted with the routine laboratory together with the samples. In case of non-compliancy, the laboratory should inform the competent authorities without delay (European Union, 1998_b).

As a summary for the European control plan, it starts by assigning the responsibility of controlling veterinary antibiotic residues to the competent authorities that are mainly ministry of health/agriculture. The tasks start by registering all

veterinary drugs to the European system through EMEA by, mutual-recognition, decentralized and centralized procedure (EMA, 2016; European Union, 2001; 2004$_d$).

Moreover, an integrated program should be established between competent authority and the European accredited laboratories working with validated methods using particular standardized equipment according to the European norms requirements, to check for non-compliancy of official collected samples (European Union, 2004$_b$).

Furthermore, only authorized drug factories and distributers that are working on a high standards level by respecting national authorities law, are allowed to produce, distribute and sell veterinary drugs. Veterinary drug dealers and manufacturers should pass through an audit system to evaluate their competence level based on the European norms. Veterinarians should respect good veterinary practices and the European norms by prescribing veterinary drugs based on the European standards after checking the animals. Prescription copies should be archived by the farmer, veterinarian and the veterinary drug pharmacy, to fulfill the requirements of any official control needs (European Union, 2001; 2019$_d$)

Maximum residue limits norms are set by the European Medicine Agency Committee for Medicinal Products for Veterinary Use (EMA, CVMP) (European Union, 2015) the Joint Food and Agricultural Organization/World Health Organization Expert Committee on Food Additives (FAO/JECFA, 2019), Codex Alimentarius (CAC) (Codex Alimentarius, 2018) and used by the competent authorities to verify the compliancy of any collected sample during a controlling procedure.

Moreover, a national plan was established to set out a base for the European member states to build up a plan to control antibiotic residues in food producing

animals based on risk assessment. This plan should be shared with the national authorities, and explains in details how the European member states will monitor veterinary drugs use. Reports for each official inspection should be shared between local competent authorities and national authorities. Control plan should also cover veterinary drugs import and export as well as food from animal origins trade between European and non-European countries. Moreover, the member states should establish a plan for screening methods and share it with the national authorities, to assure the effectiveness of their residue control program. This plan covers the sampling methods, number of sampling, transportation of the sample, frequency of inspections and monitoring method for each type of food from animal origin. Official inspections by a competent official veterinarian will be carried out in order to get samples from raw materials of food from animal origins that will be tested in the officially accredited laboratories for any non-compliancy. (European Union, 2017$_b$; European Union, 1996; European Union, 1997$_b$; European Union, 1998$_b$; European Union, 2018).

EFSA publishes yearly a report on the results from the monitoring of veterinary medicinal product residues and other substances in live animals and animal products that summarizes veterinary residues and certain substances monitoring plan results in live animals and animal products in the European Union (EFSA, 2019). The report shows that 708,880 samples were reported by 28 European member states divided in 360,293 targeted samples and 55,088 suspect samples reported under the European Council Directive 96/23/EC and of 16,542 samples collected at import and 276,957 samples collected in the framework of programs developed under the national legislation. In 2017, the percentage of noncompliant targeted samples (0.35%) was comparable to the previous 10 years (0.25%–0.37%) (EFSA, 2019$_b$).

As for the year 2016, 710,839 samples, reported to the European Commission by 27 out of the 28 Member States, were divided to 369,262 targeted samples, and 21,350 suspected samples were reported under Council Directive 96/23/EC, and 4,075 samples collected at importation and 316,152 samples collected in the framework of programs were developed under the national legislation. Overall the percentage of non-compliant targeted samples (0.31%) was comparable to the previous 9 years (0.25%–0.37%) (EFSA, 2018).

In the year 2015, 729,881 samples were reported to the European Commission by the 28 EU Member States. Divided of 411,677 targeted samples and 19,257 suspect samples reported under Council Directive 96/23/EC, and of 3,768 samples collected at import and 295,179 samples collected in the framework of programs developed under the national legislation. the percentage of noncompliant targeted samples (0.34%) was comparable to the previous 8 years (0.25%–0.37%).

More specifically, in the years 2017, 2016 and 2015 out of 10634, 11929 and 13,168 respectively targeted samples of milk collected 0.18%, 0.06% and 0.08% respectively were non-compliant for veterinary antibiotic (B1) residues (EFSA, 2017).

Moreover, in the year 2015, prohibited substances (A6) were found in 0.04% of samples. Substances identified were chloramphenicol (n = 15), nitroimidazoles (n = 9) and nitrofurans (n = 9) (EFSA, 2015$_c$). In the year 2016, 0.03% of the collected samples were contaminated with prohibited substances (A6) and divided in the following, chloramphenicol (n = 12), nitroimidazoles (n = 4) and nitrofurans (n = 10) (EFSA, 2016). The same percentage was found in 2017 but distributed as following chloramphenicol (n = 8), nitroimidazoles (n = 2) and nitrofurans (n = 18) (EFSA, 2017).

Chapter 4: Integrated approach to antibiotic residues control in milk and Dairy Chain

In order to decrease the risk of raw milk contamination by veterinary residues, an integrated system is provided. This system consists of relating quality and food safety management protocols applied by the dairy chains operators with the official controls by competent authorities, including confirmatory tests and verification program (VICH, 1999).

The dairy chain framework provides the following steps to be considered:

- Farm level (individual cow and bulk tank).
- Truck tank.
- Factory level.

At different steps self-checks by Food Buisness Operators (FBOs) and Official Control should be applied.

At each step of the dairy chain the use of different sampling strategies and tests, related to the different purposes and objectives of testing milk for residues, is required. Among test should be recognized microbiological test, rapid test and confirmatory test.

While sampling and testing, several control measure should be respected due to their critical alteration on the results outcome.

The most important control measures are listed below:

- preventive measures that should be applied (e.g. training of farmers, communication);
- the aim of sampling and testing and their main benefits;
- regulatory requirements/contract requirements;
- targeted substances and available tests that targets a specific residue;
- critical procedures for sampling and testing;
- cost-benefits of the antibiotic residues tests;
- best action in case of a non-compliant results is found and the method;
- preventing the non-compliancy repetition by tracking back the source of contamination (IDF, 2014).

A general description of the integrated approach will be explained at the four levels of the dairy chain sector.

4.1 FARM LEVEL

4.1.1 Individual cow milk

Good Farm Practices related to antibiotic residues are the proper use and registration, treated animal identification, the milking of treated animals, collecting and management of milk with residues.

Sometimes at farm level, checking cows individually in case of antibiotic treatment can prevent the contamination of bulk tank milk. It is not necessary for the farmer, but it could be a part of the contract between him and the dairy plant or a requirement for good farming practice criteria. The farmer is the only person responsible for the checks in case of non-compliancy (IDF, 2014).

Sampling is a critical point, the farmer should milk the entire udder and the test can be performed by him, the veterinarian, laboratory or milk hygiene advisor. At this checking level, a specific rapid test receptor or a broad spectrum microbiological inhibition test that can be enough to detect any antibiotic contamination can be used. In case of a positive result, there is no need to double check but checking the test kit with positive and negative control is recommended to confirm its sensitivity level. The farmer should keep in mind that sometimes false positive results may appear due to natural inhibitors presence. In such case, the farmer should discard the contaminated milk, recheck the treatment and withdrawal period of the administered drug, make sure that the test is handled appropriately and sampling conditions are correct (IDF, 2014).

4.1.2 **Bulk tank**

Official regulations do not oblige the farmer to check his bulk milk for antibiotic residues, but the farmer can perform veterinary residue auto-control in case of any doubt before bulk milk is collected by the dairy factory. By doing this checking step, the farmer can avoid the contamination of big milk quantities and prevent the negative consequences of contaminating a big amount of milk in the factory truck tank. Performing self-checks for residues also prove that the farmer is interested in delivering a good milk quality which could be a good credit for him at official and farm control level (IDF, 2014).

The farmer can use broad spectrum microbiological inhibition tests or rapid receptor test.

Sampling is a critical point; the farmer should collect a sample that represents the whole farm production. This sample should be mixed and homogenous. Tests can be performed in the field but tests requirements should be respect in term of preserving,

incubation period, temperature as well as positive and negative control usage. When a positive result occurs, the farmer is not obliged to double check, and contaminated milk should be discarded (IDF, 2014).

4.1.3 **Truck tank level at dairy farm**

Farm bulk tank are controlled by the dairy factory (this step is not applicable on field).

Checking dairy farm bulk tank before collecting the milk can prevent the contamination of a huge quantity of milk in the truck in case of residue contamination, and it helps the dairy factory control division to check if the farmer is working according to the signed contract (IDF, 2014).

Furthermore, the dairy factory control division can trace the non-compliancy directly at farm level. Testing is not an official requirement by competent authorities unless some regional official control requires this action. In normal cases, dairy collector/processor is responsible fors taking the decision to test or not (IDF, 2014).

Sampling is a critical control point and it could be done manually or automatically but it is imperative to obtain a representative sample. Tests can be performed by the dairy advisor or by an authorized person from the dairy factory part, using a broad spectrum microbiological inhibition test or a rapid receptor test (IDF, 2014).

Tests can be performed in the field but it is imperative to make sure that the test is preserved in the required condition, and performed as required in terms of incubation period, temperature and positive and negative control usage. There is no need to recheck in case of non-compliancy, but discarding the contaminated milk is necessary.

In case the milk was poured in the truck tank, the bulk milk tanker should be rejected at the dairy factory as required by the local regulations (IDF, 2014).

4.2 FACTORY LEVEL

1) Farm bulk tank is not checked before pouring it to the dairy truck therefore the risk of having a positive result in the truck bulk tank is high.

- Dairy Factory role:

Checking the milk received from the farms in the dairy trucks, helps detecting any non-compliancy before residue contamination occurs by mixing the collected milk with dairy factory silos.

This step is very important for the dairy milk plant production, it decreases the risks of discarding a huge quantity of milk in case of cross contamination, prevents any loses regarding technological level in term of processing, protects the quality and preservs the respectable image of the dairy factory products.

Depending on the country, tests could or not be under official regulations. The dairy factory is responsible of deciding whether to perform the tests or not, and only the driver or the dairy collector of the dairy factory can perform the tests while respecting sampling requirements (critical control point) by taking a representative sample.

Rapid method tests can be used, and in case of a positive result, it is advisable to recheck the milk using a test that defines quantitatively the non-compliancy in term of maximum residue limits. When the tests prove the presence of non-compliancy, milk in the truck should be rejected.

Furthermore, the dairy factory should trace-back the origin of the milk and inform the official control. Based on the Farmer-Factory contract, the farmer should respect the penalties (IDF, 2014).

4.3 OFFICIAL CONTROL ROLE

4.3.1 Official control role in case of a non-compliancy was detected at dairy factory level

The official control should visit the non-compliant farm collect milk samples and check them in a private laboratory to double check the non-compliancy. Moreover, they have to investigate the reason of the non-compliancy and make sure that the milk is free of any residues before allowing the farmer to sell it again. In some cases, where the farm has acritical history of antibiotic residues cases, the official control will close the farm (IDF, 2014).

4.3.2 Official control at farm level

Testing the bulk tank of farmers is under official regulation and governmental authorities build privately the schedule of farm-visits. Bulk tank control system improves the farmer attitude towards producing better milk quality, detect critical farmers, and enforce farmers to sell milk as required in terms of quality fulfilling official and factory requirements (IDF, 2014).

Sampling is a critical point, manual or automatic sampling can be performed by an officially authorized qualified person that collects a representative sample of the whole farm milk quantity (IDF, 2014).

Only an authorized laboratory that hires skilled qualified technicians will perform a broad spectrum microbiological inhibition test, or rapid tests. In case of non-compliancy, the sample should be checked using a chemical method to define quantitatively and qualitatively the source of contamination (IDF, 2014).

In case of a non-compliancy confirmation, the official control will ban the farm and other consequences are decided based on the local regulations (IDF, 2014).

Different examples are given, demonstrating the official enforcement of the integrated approach in different developed countries:

4.3.3 United States of America

At farm level, a representative sample should be collected by the bulk milk sampler from each farm bulk tank before pumping the milk from the farm tank to the truck or other container. At industrial level, the industry plant sampler should collect a sample from each truck milk tank, before allowing the milk to be added to the dairy factory bulk tanks. At factory level, tests should be completed before processing the milk, knowing that all farms should be checked four times every 6 months. If the test reveals a non-compliancy, the milk should be rechecked with a test which activity is confirmed with positive and negative control. In case of a double positive result, milk should be discarded. Moreover, tracking back the source of the contamination should be done to reach the milk production origin at farm level. When detected, the farm bulk tank should be checked as well. When a double positive result is obtained at farm level, the milk sample producer is confirmed as non-compliant (IDF, 2014).

4.3.4 France

A representative sample should be taken at farm level before loading milk into the truck tank and sending it to the dairy plant. A central independent laboratory will collect samples from the dairy plant and plan privately to analyze them at least 3 times per months. At the dairy plant level, a representative sample should be collected from the dairy truck and tested systematically using rapid test by the dairy collector before mixing the collected milk with the plant bulk tanks. When a positive result is found, a European approved inhibition tests are used to double check the non-compliancy. Milk is pooured into the factory bulk tanks in case of a compliant result (IDF, 2014).

In case of a non-compliant result confirmation milk will be discarded and tracing back the origin of the milk is essential. All loaded milk samples should be analyzed by an official inhibitory test and positive samples should be checked again using several receptor tests to identify the cause of contamination. Farmer responsible for the non-compliancy will be penalized and should pay for the destruction of milk that was poured with his non-compliant milk in the same truck tank (IDF, 2014).

4.3.5 **Germany**

On a farm level, sampling is done automatically according to the regulation DIN 11868, through a sampling system installed in each bulk milk tanker. Depending on federal states decisions, the frequency of samples is done and performed by a personal accredited laboratory or the dairy factory.

On the factory level, sampling can be done voluntary or obligatory based on the dairy factory rules and the federal states. When sampling is necessary, it should be done before pouring milk in trucks to the dairy plant tanks. The quantity of samples should be representative and depending on the dairy factory decision (IDF, 2014).

On a farm sampling level, tests should be done by an independent laboratory accredited by the federal state according to MilchGüV (milk quality regulation) and regulation of the federal state (MilchGüV, 1980).

According to the regulation MilchGüV, 2 milk tests should be performed per months but some federal states tests up to 4 times per month. Inhibitor tests should be used according to the regulation §64 LFGB (Food and Feed law) method L 01.01-05 (microbiological inhibitor test).

According to the regulation §3 MilchGüV, inhibitors should not be present in the milk. Based on the German Food and Feed law §64 LFGB, method L 01.01-05, a sample is defined as positive when the sample color is blue similar to the positive control (4ppb Penicillin G)). A double check using the same technique is necessary to confirm that a sample is non-compliant.

On a factory sampling level, the dairy plant should decide if there is a need for rapid or microbiological test. In case of any non-compliancy while using rapid test, the European Legislation states that the dairy plant can perform a chemical confirmatory tests to verify quantitatively and qualitatively if the non-compliancy is above the maximum residue level or reject the milk and discard it according to regulation (EC) N:1774/2002 on animal by-products (European Union, 2002).

When a positive result is obtained from a milk truck that contains a big quantity of milk originated from different farms, traceability of the source is essential, by testing samples from each farm in an independent laboratory.

When the non-compliant farm is traced, the farmer is obliged to sell his milk to the factory in a price reduced by 5 € Cent/kg according the regulation §4 MilchGüV. Moreover, the farmer will be visited by the relevant authorities and he should be able to guarantee that he is using veterinary drugs prudently. Concerning the dairy plant, in case of any non-compliant results, they can oblige the farmer to compensate for the damages and pay a penalty based on the factory-farmer contract (IDF, 2014).

4.4 PERSONAL EXPERIENCE IN SARDINIA

After completing a stage of 2 months in Sardinia, that was done in the 2 biggest cheese making plants (F.lli Pinna Industria Casearia S.p.A and Cooperativa Allevatori Ovini formaggi), a laboratory working in collaboration with the regional cheese making plants (Associazione Regionale Allevatori Della Sardegna) and a private laboratory working in collaboration with the official control (Istituto Zooprofilattico Sperimentale Della Sardegna), a clear idea about antibiotic residues control plan is understood.

The following parts of this chapter will explain the integrated approach between dairy plants, farmers and official control in Sardinia.

In order to control in field raw milk quality before processing it, a special office is established specifically and have the responsibility of buying the milk and controlling its import to the dairy factory.

Every legal farm should be registered in the ASL (Azienda Sanitaria Locale – Local Health Unit) that is the center of administrative operations related to Public Healthcare in Italy under the National Healthcare Service (SSN – Servizio Sanitario Nazional).

ASL is divided into 3 areas:

Area A: control of new farm data and registrations, animal diseases, prophylaxis, regulation of farms and animals (for the BDN: Bank Data National).

Area B: control the meat, honey, processed meat, eggs, fish, and slaughter houses.

Area C: control the behavior of farmers, transportation of livestock, registration of companion animals (canine) and milk with pharmacovigilance.

A file for every farm contains data showing type and number of animals, type of production and a registration in BDN to verify that this farm is respecting the rules of hygiene, location, number of animals, veterinary drugs usage, microbial inhibitors usage….

Moreover, a code should be given to the farmer by the ASL that will be used in every official control activity (Code of registration of ASL 852/04) (European Union, 2004_c).

When a farmer wants to sell his farm milk to a dairy factory, after collecting all data needed from Azienda Sanitaria Locale (ASL), Banka Dati Nationale Di Teramo (BDN), organization of Pecorino Romano and Pecorino Sardo production specifications (INEQ) and the ID's, the control in the dairy factory checks the papers and verifies if everything is legal. In addition, personal information of the farmer and workers such as Identity cards copies, are archived in the dairy factory control office.

It is necessary to mention that the laboratory of the Regional Farmers Association of Sardinia (ARA) is qualified in accordance with ISO 17025 standards. The lab is mainly involved in own checks on raw milk as provided by Regulation 853/2004 (European Union, 2004_d). For this activity the lab should be approved by the Competent Authorities (Regional Service of the Health Ministry) and it is included in a regional list. The data produced by the laboratory is also used by the Competent Authorities to evaluate the compliance to the requirement of the Regulation 853/2004 (mainly Total Bacterial Count and Somatic Cell Count). For inhibitors the lab is involved in the framework of the Official Control measures when not compliant (positive) samples are detected. The dairy factory milk control department can check if the farmer is registered in the ARA (Regional Farmer's Laboratory Association) by contacting ARA and ask them for the code of the farmer.

Moreover a website for ARA (www.ara.Sardegna.it) that gives access to dairy factories by entering their username and passwords was established in order to let them check for milk results that were analyzed by ARA laboratory.

If all the above steps are completed, a contract is built with the costumers, that identifies and verifies if the costumes are qualified to produce milk by respecting the rules of the regional association health authority, INEQ (organization of Pecorino Romano and Pecorino Sardo production specifications) and BDN (Banka Dati Nationale Di Teramo); (The BDN belongs to the zooprophilactic Institute of Teramo).

Moreover, dairy factories have to get a farm book explaining rules for milk production that is available at the Ministry of Agriculture, a copy of all the documents of the farmers and their farms and a copy of the rules of ASL, INEQ and BDN in order to work in compliancy with the European system requirements.

So for now we covered the documents that are legal and the code given by ASL to the farmer.

After checking that the farm is legal the dairy factory control team will start the discussion about buying the milk from the farmer. Based on the Sardinian deliberation number 53/4/del 29-12-2014, each dairy factory defines its control protocols and rules. When everything is confirmed between both parties, dairy factories establish a contract that should be signed by dairy farmers in order to sell their milk to the dairy factories. This contract lays down rules on milk quality and steps to be passed in order to make sure that milk bought from the farm is safe for food human consumption. When the farmer signs the contract, he will be responsible for any non-compliant milk sold to the dairy plant. Farmers should give milk free of contamination to dairy factories. If antibiotic residues are detected in the milk, the company will not pay money for the farmer, and the milk won't be collected anymore from the farm until the competent

authorities take control of this farm and clarify that the milk is free of antibiotic residues.

The control system manages the raw milk collection from these farms in collaboration with the truckers. A specific plan is used to collect samples of farm bulk milk for inhibitors in a proportional way covering all Sardinia's business dairy farms. Most of dairy factory that has to manage the periodic rotation as stated by the guidelines prefers to check all the farms every 15 days (when samples for assessment of other requirements of Regulation 853/2004 are taken) because the rotation program is difficult and time consuming.

Starting with a controller (trained trucker) that works in the dairy factory, he collects samples from the farms that are listed in a schedule made by the ARA laboratory. ARA will decide the number of farmers and number of samples for each dairy plant and the control of the dairy factory will decide from which farmer to collect the needed samples.

The collection technique is a critical point. Before collection, the collector should learn how to collect samples in different methods and in different cases. First of all, checking the temperature of milk and the pH is very important to verify if this milk will be used or not and to prevent mixing it with other compliant milk in the collection tank. If there is a dropper, the collector should enter the total quantity of milk and the quantity of milk pumped per minute (Liter/minute) so the machine will regulate the dropper speed to obtain a sample from the whole tank quantity.

If the collection is done manually, a specific manual mixer with a cup made from stainless steel is used. The dimension of the mixer and the cup are given by UNI (Ente Nazionale Italiano di Unificazione; Via Battistotti sassi, 11B 20133 Milano, Italia). A specific regulation is made to determine the specific sizes of the mixer and cup used

to collect manually from bulk milk tanks. The collector needs to mix the milk for 5 minutes if manually or 1 minute if there is an automatic mixer system, after that collect the sample. The quantity and number of samples collected are based on the European norm in 'UNI EU ISO 707' on December 2008. Per example, if many tanks are present in the same farm, the sampler should collect a sample from each tank than mix them all together and remove one sample from this mixture. For a 1000 Liters tank the sampler should collect 5 samples and mix them together, then collect one sample from the mixture. The materials used for collection should be disinfected properly and washed with water from disinfectant residues to prevent a positive result in inhibitor test (Delvotest). The sample collected should be labeled immediately, locked and the sampler should put it immediately in a fridge controlled on 4°C. The samples are locked in the fridge of the dairy factory controller office until the ARA controller arrives to collect samples to check them at ARA laboratory. As soon as the ARA controller arrives he will put the samples in the car prepared for the transportation of raw milk samples and he will take the papers of samples from the dairy factory where is written the number of samples, the code of the farm, and the code of the dairy factory. The car of samples transportation contains a data logger that records the fridge temperature continually. Temperature data is collected by inserting the data logger on a computer with a specific program. In this method, the ARA laboratory can detect any problem in the temperature that might indirectly affect the results of collected samples, and if some results were found positive and ARA was accused with a bad transportation, the system of the data logger can be a proof that at any specific date and time the temperature of the samples were as required by ISO 707 (ISO 707, 2008).

Samples should be tested within 24 hours, if not, then within 42 hours, but the ARA should inform the laboratories from whom the samples were sent, and in case of not testing the samples during 72 hours, the samples will be discarded.

Farmer should be sure that the samples are taken in the right method, this is why ARA has made a video that is very simple to farmers with different level of knowledge to make sure that they can understand and approve that their samples are taken in the perfect method.

Moreover, dairy factory control department should check the hygiene of the truck container before collecting milk. In addition, the water used to clean the tank with detergent will be checked at the end of the washing to check the presence of detergent residues that may appear as bacterial inhibitors in Delvotest. This procedure is made with checking the pH of the water used to wash the truck container before entering the container and from the faucet where it comes out after cleaning. When the pH is basic it means that there is still detergent residues. Washing again is important to prevent a false positive result of the collected milk because of an improper way of cleaning in dairy factory.

Each trucker has his own zone to collect milk from and each zone contain many farms that are identified by a number (it is the registration number of each farm, as assigned in the National Data Bank).

Training the trucker for sampling is a very critical job. First of all, they should be approved by the ASL not just for driving a cittern truck, but for food transportation as well. If the trucker is trained in a bad way or if the trucker is not qualified for sampling, then he is not allowed to perform sampling farms. The improper way of sampling may lead to sever problems such as cross contamination of samples, or misleading data on milk composition and other parameters (example: fat %, somatic

cell count, or total bacterial count). A false positive result is a problem for the farmer and the laboratory as well. A double sample is necessary to keep one with the farmer locked and another to give for the laboratory for residue testing.

Knowing that contaminated milk collection may lead to the contamination of compliant milk in the same tank, dairy factories milk collectors should check milk for temperature and pH (minimum 6,3). When milk is compliant, a sample should be collected based on ISO 707 (ISO 707, 2008) in a specific cup that will be locked (European Union, 1997$_b$) and labeled with the farm code (e.g. Codes: IT 023 SS 044 per example is an identification code and means Italy district 023 Province Sassari zone 044) and milk will be collected to the truck tank in the presence of the farmer.

When trucks arrive to the dairy factory, an electronic machine is used to enter the name of the farmer or the farm code and the quantity of milk collected based on a digital counter that counts the quantity of milk emptied from the truck to the dairy milk tanks. These data will be entered to the system to compare the quantity of milk given by the trucker and received in the dairy factory to prevent any milk steeling during the transportation. A double check on the milk quantity is performed. To prevent any conflict, a small booklet is left with the farmer where the trucker writes down the collected milk quantity in his farm. So a triple check system is used to prevent any quantity problems, the farmer knows the quantity of milk sold and the dairy factory with the truckers know the received quantity.

Before sending the approval to the milk receiving center in the dairy plant, the collected samples from the farm are sent to the internal dairy plant laboratory to verify any non-compliancy. Two samples from the truck bulk milk are collected in blue plastic containers based on ISO 707 norms. One to the dairy factory internal laboratory and another one for ARA laboratory that in its turn collects their samples from the

dairy factories in specific cars with controlled and monitored temperature to transport them from the factory to their laboratory.

Moreover, there is also a contract signed by the lab and the official authorities that allowed to the latter to use the same data to assess the compliance of the results of the analysis to the requirements of the Regulation 853/2004 (Total Bacterial Count) and to some requirements of support measures for the farmers financed by Sardinian Region and European Union.

Sampling cups that will be sent from the internal dairy plant to ARA laboratory are color coded. Pink is for goat milk, green for sheep milk and white for cow milk. Moreover, samples containers should maintain samples quality, prevent cross-contamination and degradation and be officially sealed and suitable to maintain traceability and integrity. The sample quantity should be enough to perform complete screening and confirmatory analysis procedures by the approved laboratories. Samples should also be divided into equal sub-samples (at least 2) that each one is enough to perform the analytical procedures needed, unless the national legislation does not require the sample division or the sample cannot be divided technically. All samples should be monitored and tracked. This is why they put a sticker on each sample on which is written the serial number of the laboratory sending the samples and the serial number of the farm that produced this milk with a bar code for all the information on every sample for an automatic entry system. A special form should be filled with dates and all number registered on samples (European Union, 1997$_b$).

When received, the blue cup labelled samples will be identified by the dairy factory laboratory. Then, milk sample temperature and pH is tested using a calibrated pH meter and thermometer. The pH should not exceed 6.3. If it is under the required limit, the milk will be rejected. If the pH tested by the trucker at the farm was good

and the collected sample reaches the factory laboratory with a pH less than 6.3, another sample is collected from the truck to double check if the sample was not stored in a good condition. After checking the pH and the temperature of the sample, a rapid test for detecting mixed milk from different species is performed. Per example, a test allows the detection of traces of cow and goat milk in sheep milk (Prognosis Biotech, rapid test Goat; Prognosis Biotech, rapid test cow). It is a test of 3 minutes. It requires adding 4 drops of the buffer solution to a small beaker with 2 drops of the sample. Than putting a strip for goat milk detection and another for cow milk detection will allow to detect the traces of different milk present in the same sample. After 3 minutes, if 1 line appears which is the control line (highest line) that means the results are negative and there are no traces of mixed milk. But if 2 lines appear, it means that the milk is mixed with cow or goat milk (depending on the strip used, if a cow strip for detection of cow's milk and if goat strip for detection of goat milk). This test is carried out because of allergens control (example: cow's milk could be more harmful for people that are sensitive) and to prevent fraud. The current legislation considers a fraud if the milk of different animal species is mixed with the one that is declared in the label. The same occurred for PDO cheese (as Pecorino Romano) a regulated qualification that protects the designation of origin in European Union countries (Regulation (European Union, 2012).

After checking for mixed milk, checking the antibiotics residue is an imperative step before allowing milk transfer from trucks to dairy factory tanks. Certified rapid tests are used to detect in a fast qualitative method the presence of antibiotic residues above the maximum residue limits. Twin sensor rapid test is one of the tests used to identify in 6 minutes the presence of beta-lactam and tetracycline residues in milk. The tests contain a small beaker with powder. You mix milk with the powder after putting

the beaker in a special incubator on 40°C temperature. After 3 exact minutes, you emerge the strip sponge and after another 3 minutes you can read the results. The strip contains a control line. It appears with pink color. Above this control line we have the tetracycline detection line and below the control line we have the B-lactam detection line. If 3 lines appear (pink color of the lines for antibiotics should be more dark than the control line) than the sample is negative for any residues. The line that does not appear means a positive result of contamination of the milk. If you have a false positive or a false negative result (the pink is not clear enough) the sponge part of the strip is removed and the strip is placed in a Sensor Reader (strip reader) and the reader will identify the result if it is positive or negative.

If the milk sample is compliant with the European standards, milk will be pumped into the dairy plant tanks. Not to forget that the internal dairy plant and ARA laboratories will check identical samples from the same milk source.

Once a non-compliant result is detected, they should inform the official control about the non-compliancy, otherwise, the laboratory is considered subjective in his checking activities (European Union, 1997$_b$).

Farmer responsible for the non-compliancy will not get payed for his non-compliant milk that will be discarded and he will be responsible of a penalty based on the signed contract between the farmer and the dairy factory (European Union, 1996).

In case of non-compliancy, samples will be collected daily from the farm responsible for the non-compliancy by the laboratory inspector and the official control investigator and tested again until antibiotic residues are no more detected. Moreover, official control investigators should check the reason of the antibiotic residue in milk and using veterinary drugs tractability system, prescriptions and the farmer booklet where all administered antibiotics are recorded, to identify the cause of the non-

compliancy. Depending on that cause, official control defines the type of penalties set for the case (European Union, 1996).

When results appear negative, the farmer can sell his milk again. If antibiotic residue is suspected for 2 consecutive times per year for the same farm, the farmer will get a penalty and the factory won't collect milk anymore from the farm.

Based on its compliancy or non-compliancy, a strategy for every sample results checked in ARA laboratory will take place. For instance, a positive result should be double checked. When a double positive result occurs, an automatic e-mail will be sent to the dairy factory and Competent Authorities with the code of the sample. When a negative result occurs after a double check, an email will be sent to the dairy factory in order to pay more attention to the farm responsible of this sample.

All results will be uploaded online with a username and password, so each dairy factory laboratory that sent a sample for ARA can check for the results uploaded by ARA laboratory with an accessible results history starting the 1st day.

On the online software, results appear as ++ (double positive), ± (trace), -- (negative).

If they find a double positive result, ARA laboratory will contact the private laboratory of the dairy factory and the official control, but they don't contact the farmer. The official control sends a veterinary controller every day to the non-compliant farm to investigate the reason of antibiotic presence in the milk and samples will be collected for 3 successive days. When samples are double negative then they contact the dairy factory to recollect the milk from that farm. (ASL can put penalties for the farmers that have residues in milk depending on his history and risks based

assessments). Somatic cell count (SCC), inhibitors and bacterial count are critical parameters for ARA laboratory.

When milk is free of antibiotic residues and is allowed to be stored in the dairy plant tanks, samples are checked for the freezing level and bacterial growth inhibitors.

Delvotest is mostly used as a microbiological test to check for bacterial growth inhibitors (DSM, 2019). The color of the tests is purple. You add milk to the small cups of the test that contains a special gel and you incubate it at a temperature of 64°C for 3 hours. If the gel remains purple, it means you have positive results of antimicrobial growth inhibitors (DSM, 2019).

All Delvotest positive samples should be double checked. If you obtain a double positive test you need to contact the farmer and the Competent Authorities as well (European Union, 1997$_b$).

The freezing level of milk and added water to the milk should be checked as well using a calibrated cryotest machine. You put 2 to 2,5 ml of milk in a special beaker, then you put it in the machine to collect data.

The dairy plant control department should build a schedule for unexpected checks in which the control person will collect samples twice per month arbitrarily from each farm that has signed a contract with the dairy plant (Regulation 853/2004). Samples containers are color coded as described before and should maintain samples quality, prevent cross-contamination and degradation, officially sealed and suitable to maintain traceability and integrity with enough quantity to perform complete screening and confirmatory analysis procedures by the approved laboratories. On each cup the private laboratory should put a sticker to identify his name with the number of the

dairy factory and the ID of the farm that is based on HACCP code. Example: IT 023 SS 044 (European Union, 1997b).

Recording the samples results should cover the name of the farm with positive or negative results time of receiving the sample, the time of checking and the signature.

All official documents should be written on a paper where a specific place is available for seals and signatures.

When a positive sample occurs in the ARA laboratory or in an auto- control laboratory of a dairy factory, or when the schedule of the official authorities confirms a sampling for the control strategy, the samples are collected from a Veterinarian working with the official control in the proper method described by ISO 707 and sends it to the "Instituto Zooprophilattico Sperimentale della Sardegna", to confirm the primary results. This experimental zooprophylactic institute carries out experimental veterinary scientific research, animal health assessment and health of animal products. It is accredited with new machinery and a high technology and accuracy system. The Region defines the general objectives, priorities and guidelines for the activity of the experimental zooprophylactic institute in health planning, including the arrangements for liaising with the ASL Prevention Departments, the Regional Health Agency, with the Regional Agency for the Protection of the Environment of Sardinia (ARPAS), as well as with the establishments or companies developing and experimental animal husbandry research on the regional territory.

The Experimental Zooprophylactic Institute contributes to the functioning of the Regional Veterinary Epidemiological Observatory. Moreover, Zooprophylactic Institute is incorporated under the Integrated Regional Health Information System (SISAR) and plays a major role as scientific and operational necessary support for veterinary police actions and carries out any other tasks of veterinary interest to be

conceded out by the Region or by the State, including collaboration with the Community Veterinary Officer's Offices (UCACs) and the PIFs.

When official samples reach the Zooprophylactic laboratory, they are coded with serial numbers to respect the privacy of the source and prevent any interests of conflicts. When the sample arrives to this laboratory and there is no possibility to check directly the samples, they are kept in a refrigerator at a temperature of -20°C. If there is a possibility to check directly the samples, they are sent to the antibiotics residue department. Beta lactam, sulfa and macrolides are checked with Delvotest. Quinolones are checked with ELISA and tetracycline is checked with twin sensor rapid test. In case of positive results, a chemical test (Mass spectrometry and High-performance liquid chromatography) will be performed to confirm the results. Approximitely two days are needed to obtain a final result.

When the laboratory finds a positive residue sample and after it is confirmed, it is the laboratory's responsibility to inform the official control during 24 hours from finding the non-compliant result.

An e-mail will be sent including the name of the farmer, name of the dairy factory and the codes of both to the official control.

The official control is divided into 3 parts, A, B and C.

A) control animal sanitary (Farmer).

B) control the hygiene of the alimentation.

C) control the production of food from animal origin from farmer to consumer.

Based on the 'REGOLAMENTO (CE) N. 882/2004 DEL PARLAMENTO EUROPEO E DEL CONSIGLIO del 29 Aprile 2004' the community of the official control assigns a specific person who is responsible for controlling the system in a

particular area with a precise job description that is classified in details with the name of each person responsible of this job. All these laws are under the regulations of the Ministry of Agriculture in Italy. For Sardinia, a specific autonomous control system is formulated, it is personal and it refers to the European Regulations 822/2004.

In the Sardinia autonomy control system, the objective 3.2 'Procedura di notifica e gestione delle non conformità per presenza di residui in sostanze inibenti e di altri residui e contaminanti riscontrati in autocontrollo' explains the way of controlling inhibitors and antibiotic residues in food from animal origins such as milk.

The official control sends an official veterinarian to the farmer to investigate for the presence of antibiotic residue in the milk farm. To check the booklet of the farm that belongs to it where the name of the veterinarian that has described the medicine, the description of the case, the number of the treated animals, the serial number of each treated animal, the code of the medicine and the dosages with the remaining quantity at the end of the treatment are present.

Not only to check the book but also the prescription of the private veterinarian that prescribed the medicine. This prescription has 4 color coded copies, one with the veterinarian, one with the pharmacy, one with the farmer and one with the official control (blue). In this prescription they can find the description of the case with number of animals treated with the serial numbers of each animal, the duration of treatment with the dosages and the farm code. After verification and finding the cause, the official veterinarian writes a report concerning the farm investigation findings and restrict milk usage for 3 consecutive days. During those 3 days the dairy factory and the official control should check the non-compliant milk to guarantee that after 3 days the milk is free of residues and/or does not exceed the maximum residue limits. After

3 days, if the residues remain higher than the maximum residue limits, the milk will be discarded until the official laboratory assume the negative results.

Concerning penalties, they depend on the contract between the dairy factory and the farmer as well as the farm history and its risks based assessments.

Chapter 5: Controlling veterinary antibiotic residues in Lebanon

5.1 GOVERNMENT LEVEL

In Lebanon, Food Safety Law was approved and published on 24th of October 2016 by the Lebanese Parliament as Law No:35 (Cortas, 2017).

The Ministry of Agriculture plays an important role in the implementation of Food Safety Laws and Decisions. Registration, importation, manufacturing and handling of veterinary medicinal products, disinfectants and sterilizers as well as veterinary raw material, and feed additives rules are established in the Ministerial Decision No: 121/1 on 27th of January 2011. Moreover, regulating veterinary sales and prescription was explained in the Ministerial Memo No: 112/1 established on 21st of December 2010.

The Lebanese Official Control, defines veterinary medicinal products as "chemical or biological substances used in animals, poultry, aquatic animals, bees and birds, including ornamental birds for the prevention and topical or systemic treatment of diseases, epidemics and harmful parasites" (Decision 121/1, 2011). Moreover, the

Decision 121/1, 2011 has divided the veterinary medicinal products in the following two groups.

Group one:

a. veterinary medicines used to treat any medical condition or biological disorder in animals, or to prevent animal diseases;
b. veterinary medicines used to control internal or external parasites;
c. vitamins, minerals and other nutrients used for treatment through injections or drinking water;
d. chemical and biological substances and products used to protect or treat beneficial insects such as bees;
e. veterinary vaccines used to prevent animal diseases;
f. serums used to treat some animal epidemics.

Group two:

Medicinal feed additives containing antibiotics and/or anti-coccidials. Some antibiotics are included, as Bambermycins / Flavophospholipol, Tylosin phosphate, Virginiamycin and Avilamycin. Anticoccidials are Decoquinate, Diclazuril, Halofuginone hydrobromide, Lasalocid sodium, Maduramicin ammonium, Monesin sodium, Narasin alone, Narasin / Nicarbazin, Nicarbazin alone, Robenidine hydrochloride, Salinomycin sodium and Semduramicin sodium.

The Decision 121/1 is published and communicated immediately after its issuance in the Official Gazette and enforced since 7th April 2011.

5.2 REGISTRATION REQUIREMENTS FOR VETERINARY MEDICINAL PRODUCT MANUFACTURERS

In order to register a veterinary medicinal product, some requirements are needed from the manufacturer to fulfill the Lebanese official control requirements. First of all, veterinary medicinal product manufacturers are obliged to be registered at the Department of Animal Health/ Directorate of Animal Resources before it is allowed to register their veterinary products (Decision 121/1, 2011).

This registration is done by submitting an application form as well as a file enfolding a duly authenticated certificate issued by the competent authorities in the country of origin, proving that the company has an official authorization to manufacture veterinary medicinal products in the country of origin including the license number and the date of the authorization. Moreover, the manufacturer must be certified by the local competent authorities or international certified corporations with Good Manufacturing Practice (GMP) or an equivalent certification.

Furthermore, the Directorate of Animal Resources shall submit the manufacturer's registration application to the National Committee for Veterinary Medicines and Vaccines which will decides whether to approve the drug or deny registration or request file completion.

5.3 VETERINARY MEDICINAL PRODUCT IMPORTERS

As Provided by Decision 121/1, (2011) to import veterinary drugs, all importers should be registered at the Ministry of agriculture under the responsibility of the technical manager of the company, institution or warehouse. The technical manager should be only a full-time veterinary doctor or pharmacist.

Moreover, applications for the registration of companies, warehouses and establishments wishing to import veterinary medicinal products shall be submitted to the Department of Animal Health at the Directorate of Animal Resources with the following documents:

- the application form for the registration of the company, warehouse or establishment attached to the decision 121/1as recommended by form no: 3;

- a certificate of registration in the commercial register or commercial certificate;

- a real property certificate or lease contract;

- a cadastral map of the warehouse and its annexes;

- a copy of the technical manager's identity card (veterinarian or pharmacist);

- a copy of the technical manager's university degree, practicing license, and union membership certificate;

- a copy-conform of the contract of employment concluded between the importing establishment or company and the technical manager (veterinarian or pharmacist).

When documentations are completed, a committee composed of the General Director of Agriculture, as chair, and the Director of Animal Resources, the Head of the Animal Health Service, the Head of the Import and Export Service and the Veterinary Drugs Officer designated by the Minister, as members, will check the compliancy of the registration applications (Decision 121/1, 2011).

This committee should report to the Minister of Agriculture the final approval within fifteen days of submission of all documents. Furthermore, warehouses,

companies and establishments licensed to import shall be subject to the sanitary conditions attached to the Decision No 121/1 under Form No. 4.

Moreover, all companies, establishments or warehouses that import veterinary medicinal products should be licensed to import and store the substances mentioned based on their classification in Group One and Group Two of the Decision 121/1 Article 1.

5.4 REQUIREMENTS FOR THE REGISTRATION OF VETERINARY MEDICINAL PRODUCTS

In order to register veterinary medicinal products, companies or warehouses shall apply by submitting the following documents:

- a registration application form for each of the veterinary medicinal products (local or international), to be obtained from the Directorate of Animal Resources, in accordance with Form No. 5 of the Decision 121/1;

- a duly authenticated Drug Registration Form in accordance with Form No. 6 of the Decision 121/1 for local and international veterinary drugs;

- a duly authenticated free sale certificate proving that the product is sold in the country of origin, except for veterinary vaccines and medicines that are made or produced in a country free of the associated diseases, or a Certificate of Pharmaceutical Product CPP or Certificate of Medicinal Product issued by the European Medicines Evaluation Agency (EMEA) for medicines imported from a European Community member state;

- a certificate of analysis of local and international veterinary medicinal product listing the physical specifications and active ingredients, or adjuvants, in the case of vaccines;

- the methods of analysis of Group One medicines (except Veterinary vaccines used to prevent animal diseases and serums used to treat some animal epidemics) and Group Two medicine of Article 1 of the Decision 121/1, together with the chromatograms of the materials analyzed by chromatography (local and international drugs);

- three samples of each type of the local and international veterinary medicinal products provided that the sample size does not exceed 1 kg or 1 liter to be analyzed in laboratories approved by the Ministry of Agriculture. The product package shall be labeled with an indication of the production date and expiry date;

- the technical file of the local and international veterinary medicinal product including the stability data, storage conditions, pharmacological and clinical data, toxicological data and side effects, withdrawal period;

- a CD containing the product technical information, attached to the registration application form of every local and international veterinary medicinal product;

- a statement issued by the competent authorities in the country of origin proving that the vaccine manufacturers comply with the country's approved pharmacopoeia, such as the standards and specifications of the European Union or the European Pharmacopoeia, the US Department of Agriculture USDA, the Food and Drug Administration FDA or the World Organization for Animal Health (OIE);

- the National Committee for Veterinary Medicines and Vaccines may decide to exclude certain types of feed additives that contain antibiotics that is not used in the

country of origin, at the request of the importing company and pursuant to a scientific investigation report issued by the Directorate of Animal Resources (Decision 121/1, 2011).

After registering any veterinary drug and vaccines, the National Committee for Veterinary Medicines and vaccines should establish a decision authorizing or denying such import (Decision 121/1, 2011).

In order to renew an approved registration, the same procedure should be done every 5 years by submitting the following documents: a free sale certificate, a certificate of analysis and methods of analysis, a good manufacturing practice GMP certificate, a CD containing the product technical information (Decision 121/1, 2011).

5.5 PRE-AUTHORIZATION REQUIREMENT TO IMPORT VETERINARY MEDICINAL PRODUCTS TO LEBANON

Importing veterinary medicinal products to Lebanon should start by submitting of a pre-authorization application form, in accordance with the Form No 7 attached to the Decision 121/1 available at the Directorate of Animal Resources of the Lebanese Ministry of Agriculture (Decision 121/1, 2011).

The pre-authorization to import is effective for 6-month period from the date of its issue. This pre-authorization application form should be submitted and attached with the following documents:

- a proforma invoice issued by the company from which the veterinary medicinal products will be imported. The invoice should include a description of the items to be imported, their quantity, packaging, import price, manufacturer's name, country of origin, country of provenance, and border crossing-point.

- detailed information on the distribution of the previous shipment of veterinary medicinal products to the distributors, presented in a table signed by the company concerned in accordance with the Form No 8 attached to the Decision 121/1.

- a copy of the certificate of analysis of the items to be imported, listing their physical specifications and active ingredients.

Moreover, in the Decision 121/1 Form No. 4, Sanitary Conditions Applicable to Warehouses of Veterinary Medicinal Products, Feed Additives, Disinfectants and Sterilizers, Raw Material, and Stores that Sell Veterinary Medicinal Products is laid down (Decision 121/1, 2011).

Warehouses should be dedicated to the sale of human and / or veterinary medicines, vaccines, serums, veterinary products, laboratory products, and animal products. The dimension of the warehouse should be at least 30 square meters or big enough to accommodate veterinary drugs authorized by the Ministry of Agriculture to import or manufacture. In addition, the warehouse should be organized, clean, not connected to any dwelling, property, pharmacy, clinic or any commercial store, isolated from heat absorption, rain-water, leakage and properly ventilated with an air conditioner in order to keep the warehouse temperature below 25 degrees Celsius. The floor of the warehouse should be smooth and free of cracks and holes and walls should be oil-based painted (minimum of 2 meters height). Veterinary medicines should be stored in shelves or cabinets with a minimum of 15 cm high from the floor level. As for vaccines, a refrigerator is necessary. All parasitic pesticides and toxins should be stored in a specific cabinet or store. Veterinary drugs should be away from the sunlight, and for spoiled and expired drugs, the warehouse should have a specific place to store them until sanitary disposal. Alternative power sources and emergency exit are

mandatory in every warehouse with a sign plate affixed on top of the front entrance showing the veterinary warehouse owner and type of business (Decision 121/1, 2011).

Drug distribution is a very important step to control. This is why, the Lebanese authority obliges warehouses not to sell veterinary drugs for treatments, vaccination, serum and other products unless the buyer have a prescription issued by a licensed veterinarian stating the dose, period and method of treatment.

As for the stores that sell veterinary medicinal products, they should be at least 16 square meters and divided into a display and storage part. Walls should be painted with oil-based paint to a minimum height of 2 meters. Veterinary drugs should be stored on clean shelves and cabinets that are at least 15 cm above the floor, and not exposed to sunlight. In addition, a refrigerator specified for vaccines and drugs requiring cold storage, should be available in the selling point with a 24/7 electricity. Moreover, a very important point is listed in the decision 121/1 stating that all veterinary drugs should only be sold for farmers holding a veterinary prescription that states the quantity of drug to be administered, period of the treatment and the method of administration.

5.6 REGULATING THE SALE OF VETERINARY MEDICINES

The Lebanese government has laid down in the Decision 112/1, rules to regulate veterinary medicines sales. In this decision, the Lebanese Competent Authority states that only veterinarians and pharmacists are allowed to sell veterinary medicines. As for the agricultural engineer, only those specialized in animal production and holding a Master's degree in Poultry Science, are allowed to sell veterinary medicines and vaccines pertinent exclusively to poultry.

Any person allowed to sell veterinary medicines should present a registration application with the Animal Resources Directorate-Animal Health Service and encloses therewith the following documents: (Decision 112/1, 2010).

1- For veterinarians:

- an application form;
- authorization to practice veterinary medicine in Lebanon.

2- For agricultural engineers:

- authorization to practice agricultural engineering in Lebanon;
- a certificate attesting to their specialization in animal production with a master's degree in poultry sciences.

3- For pharmacists:

- authorization to practice pharmacy in Lebanon;
- pharmacy License.

4- For companies or warehouses facilities:

- an agreement entered-into with a veterinarian or a pharmacist and authenticated by a notary public, or an agreement entered-into with an agricultural engineer, animal production specialist, holding a Master's degree in Poultry Science, and authenticated by a notary public, to sell poultry medicines and vaccines;

- authorization to practice the profession of a veterinarian, pharmacist or agricultural engineer in Lebanon;
- registration certificate extract from the Commercial Register.

When the license to sell veterinary medicines is obtained, it will be granted for two years and could be renewable (Decision 112/1, 2010).

5.7 VETERINARY ASSOCIATION LEVEL

The veterinary association relies on the personal Good Veterinary Practices of every veterinarian working in field, to ensure a prudent antibiotic use, knowing that there is no control system to verify the prudent usage. Unfortunately, there are no regulations or decisions issued by the veterinary association, forcing veterinarians to use prescriptions when prescribing a veterinary medicine.

5.8 DAIRY SECTOR IN LEBANON

The Lebanese dairy sector is mainly composed of Friesian Holstein dairy cattle that are raised in zero grazing intensive system. The area of the dairy farms in Lebanon is formed of different sizes that are landless, and 90% of all farms are between 0.1 ha and 4 ha with an average farm size less than 1.5 ha (IFAD, 2017).

The population of dairy cattle was estimated in 2017 by Food and Agriculture Organization of the United Nations, of about 81,262 dairy cows raised in 12,594 Lebanese dairy farms that are officially registered in the Ministry of Agriculture (FAOSTAT, 2017).

The production system is formed of 3 types that are the smallholder system, medium size intensive system and the large industrial system (IFAD, 2017).

The most dominant system is the smallholder semi-intensive system. This type of farms is formed of 1 to 9 cows with an average production of 3.750 litres of milk per cow per lactation. The production of the smallholder's semi-intensive system is based on short and informal market. Feeding system is based on concentrates and dry hay. It is found abundantly in the North, the South and Mount Lebanon.

The medium size intensive system is composed of 10 to 40 dairy cows with a high production that is usually sold to the industrial processors. The feeding system is composed of concentrates and conserved silage. This system is usually found in the Bekaa Valley and Baalbek-Hermel.

As for the large industrial farms, they are composed of several hundred (up to 3500) of dairy cows with a high production system that is used in their dairy processing plants. The feeding system is mainly formed of conserved silage and concentrates (IFAD, 2017).

Large farms type is a free-stall dairy barn with a milking parlor and the traditional farming system type is a tie-stall dairy barn where cows are milked using a pipe milking. In general, forages and concentrates are not produced by the farmer resulting in high feeding cost (IFAD, 2017).

Approximately each farm contains an average of 6.5 cows. These small number of cows per farm shows how heavy are the technical and social challenges that are fighting the Lebanese agriculture sector such as a low agricultural productivity, high production cost, poor organization of farmers into cooperatives and associations, and vulnerable groups prone to poverty, especially youth and women. Moreover, Lebanon is a high middle income country, and in the year 2017, 28.6% of Lebanese families were classified as poor and 8% of them are extremely poor below the poverty line

(2.40 USD/person/day) (IFAD, 2017). Nowadays in 2019, the poverty level is increasing and the number of unemployed people is higher than it was in 2017.

The dairy farming competition remains in the adequate feeding, especially in smallholder producers. The feeding cost of dairy cattle is around 80 to 90% of the milk production cost and about 60 to 80% of the milk price at the farm. This is why the farmer knowledge should be good enough to reduce the feeding costs. Unfortunately, traditional farmers, mainly smallholders cover the highest number in the dairy farm sector and have a low knowledge levels that leads to an improper feeding system and affect animal's immunity, a decrease in the production and milk quality (IFAD, 2017).

Moreover, old husbandry practices (Natural insemination), old equipment leading to cross contamination and improper animal handling can affect as well the production system (IFAD, 2017).

Furthermore, in rural areas youth are involved in livestock farming systems when their families are engaged in such activities. The less employees are available and poorer the family is; chances are higher for youth to be involved in the family business. In 2017, only 12% of cow famers are under 35 years old, proving that youth are no more interested in livestock business (IFAD, 2017).

When talking about the quality, milking activities plays a big role. Portable milking machines are commonly used in small to medium dairy cattle farming system (IFAD, 2017). Moreover, those milking machines require calibration and maintenance to increase comfort level of the cow leading to a higher level of milk production. In addition to poor hygiene, when the milking machine is not calibrated, mastitis cases will increase reaching 20 to 30%, affecting milk quality and yield (IFAD, 2017).

Dairy fresh cow milk production in Lebanon represents 95% of the whole milk production in the country (IFAD, 2017). It was estimated around 190,445 tons in 2017 based on FAO statistics and produced permanently all over the year. (FAOSTAT, 2017). There is no correspondence with the Ministry of Agriculture statistics which estimates the yearly dairy milk production about 390,000 tons. The Ministry of Agriculture relies on a fomula to calculate this number. This formula multiplies the average production of dairy cow milk in 305 days by the average number of lactating cows in Lebanon which is 60000 cows (equation 1).

Equation 1: $$x = (Average\ producti\ on of\ dai\ rycows\ i\ nLebanon)6.5\ tones \times (Average\ number\ of\ lactati\ ngcows\ i\ nLebanon)60000 = 390000\ tones\ [of\ dai\ rymi\ lkper\ season\ (305\ days)]$$

Local dairy products are well demanded in Lebanon with a consumption per capita 114 liters (IFAD, 2017).

The biggest production part of the Lebanese dairy milk production is promoted by the informal and semi artisanal/small scale processing sector. This production is not affected directly by the strong competition from milk powder imported by the six largest factories that are Taanayel Les Fermes/Bonjus, Dairy Khoury, Liban Lait, Dairy Day, Dainka Dairy, and Jdita (IFAD, 2017).

The high influx of refugees into Lebanon, has increased dramatically the dairy products consumptions, especially products that are processed traditionally (IFAD, 2017).

Lebanese dairy farms production data as received by the Ministry of Agriculture which is around 390,000 tons per year, is not enough to satisfy consumer demands in terms of quality and quantity, obliging the country to import almost half of the quantity

needed, estimated 348 million USD composed of 60%, 30% and 10% of cheese, milk powder and butter respectively (IFAD, 2017).

Smallholder farmers producing around 10 to 20 kg/cow/day sell their milk for around 0.70 USD per Kg and even less, but the production cost is absorbing 70% approximately leading to a decrease in milk quality in order to compensate the expenses (IFAD, 2017).

In rural areas, home-based artisanal production take place in small farms, factories or farmers houses using basic utensils with variable levels of hygiene where milk is used after pasteurization to produce yogurt, labneh and fresh white cheese. Otherwise in urban centers, consumers ask for industrial production such as UHT milk and imported cheese wiling to obtain a better quality and products variety (IFAD, 2017).

5.9 FACTORY LEVEL CONTROL OF THE ANTIBIOTIC RESIDUES IN RAW MILK

Dairy factory in Lebanon vary in the production quantity. Those factory owners own their dairy farms and buy milk from other medium size intensive system farmers in order to fulfill consumer's demands at market level.

Dairy trucks are used to collect milk, some of them are specialized for raw milk transportation and used for long distance transport, but when talking about small distance transport, unspecialized truck are sometimes used and in some cases, farmers drives their raw milk production to the dairy plants using some plastic tanks or small jars of stainless or plastic that are not isolated to preserve milk temperature.

At the farm collection point, when talking about big milk industries, milk is checked for its temperature and pH before collecting.

At the dairy plant unloading point, few data are available for the industrial control system. The biggest dairy plants are equipped with a small laboratory for quality control. The largest dairy industries check milk using lateral flow rapid test for beta-lactam and tetracycline. Those checks are necessary in all dairy plant aiming to or already have HACCP certificate that is mandatory in the General Principles of Food Hygiene, in which the dairy plant should have a control system to prevent any chemical contamination that is not allowed for human consumption (Codex Alimentarius, 2003).

No data are available proving that milk contaminated with antibiotic residues is discarded or that the factory has informed the official control to investigate the reason of the non-compliancy. Moreover, some of the biggest dairy plant industries hold ISO 22000 certification. In medium and small size dairy factories, no tests are performed to check for antibiotic residues.

5.10 COMPARATIVE DESCRIPTION BETWEEN CODEX ALIMENTARIUS AND LEBANESE LAW

In the Lebanese law, few decisions are available and are rarely similar to the Codex Alimentarius. The control of veterinary drugs is applied in the Lebanese laws as well as in the Codex Alimentarius, but on the European level, the system is more specific due to the risk profile and risk analysis approach.

The Regulation (EC) No 470/2009 sets out the maximum residue limits (MRL) in the Eurpean Union, whereas in Lebanon, no maximum residue limits are established.

The classification of veterinary drugs in the Codex Alimentarius is divided into two tables of which the first one shows drugs that are allowed for food producing

animals with their MRL and target species and the second one lists all banned drugs for food producing animals because they are a form of hazard when ingested (European Union, 2009; 2010$_b$).

Antibiotics in feed are allowed in Lebanon but it is banned in the Codex Alimentarius. Moreover, veterinary groups are divided based on different approaches. For instance, the Codex Alimentarius divides veterinary drugs based on their hazardous effects on consumers, but it is not the case in the Lebanese law veterinary drugs division.

The Codex Alimentarius obliges the European countries to control veterinary antibiotic residues. The European Union Council Directive 96/23/EC lays down the basis of establishing a residue monitoring plans, sampling frequency and range of substances listed in annex I to be tested (EuropeanUnion,1996). In the Lebanese law, no plans are established to control antibiotic residues in food from animal origins.

Furthermore, Codex Alimentarius lays down rules to control manufacturing, selling and prudent usage of veterinary drugs. The European parliament and the council of the European Union has established the article 123 in the regulation (EU) 2019/6 obliging the competent authorities to carry out a control system on the following parts; “manufacturers and importers of veterinary medicinal products and active substances; distributors of active substances; marketing authorization holders; holders of a wholesale distribution authorization; retailers; owners and keepers of food-producing animals; veterinarians; holders of a registration for homeopathic veterinary medicinal products; holders of prohibited veterinary medicinal products and any other persons having obligations under the European Union regulation (EC) 2019/6” (European Union, 2019$_a$).

As for the Lebanese law, the requirement for authorization includes the technical file of the veterinary medicinal product, a certificate of analysis of the veterinary medicinal product, a certificate of Medicinal Product issued by the European Medicines Evaluation Agency (EMEA) for medicines granted by a European Community member state. Moreover, three samples of the product (labelled as ready to be sold), provided that the sample size does not exceed 1 kg or 1 liter, to be analyzed in laboratories were approved by the Ministry of Agriculture.

As for the marketing and selling, the Lebanese law lays down rules to the veterinary drug authorized dealer to provide a safe condition for medicines. Only sanitary rules are applied but no rules are related to the record keeping of sold drugs.

Moreover, the Lebanese law provide that veterinary drugs can only be released by a licensed veterinarian's prescription stating the quantities to be administered, the days and methods of treatment (Decision 121/1, 2011). No laws are established to control veterinary prescription at any level of the system.

The Codex Alimentarius prescriptions are necessary and veterinary drugs could not be sold without them. Furthermore, the official control should regulate prescriptions through their prescription-copies and check if the drug is used prudently. The wholesale distributer and the veterinary drug retailer should keep records concerning the prescription and sold drug. The prescription system is controlled on farm, veterinary and pharmacy levels by the official control (European Union, 2019$_b$).

Many differences are clear between the Lebanese law and the Codex Alimentarius. The Lebanese law is more general than the detailed Codex Alimentarius that covers all parts of veterinary drugs production, import, export, sale, residue checks and use. Not only but the Lebanese law is very general concerning the prescription system that is very specific in the Codex Alimentarius requirements. The same goes

for the national control plan for veterinary residues that is mandatory in the Codex Alimentarius whereas there are no plans to control antibiotic residues usage in Lebanon.

5.11 ACTUAL WORKING CONTROL SYSTEMS IN LEBANON

Nowadays, the control system in Lebanon is focusing on the import of veterinary drugs and sale points authorization. As for controlling veterinary antibiotic usage, there are no control measures in field.

Veterinary antibiotics are sold without prescriptions to any person that asks for them, whether he is a farmer or not. Furthermore, no plans are available to check the prudent use of veterinary drugs. Not only but sometimes the farmer does not respect drugs usage and requirements, affecting the quality of milk production and the animal as well.

As for the residue controlling system, the official control does not check for any residues in raw milk at farm level neither at factory level or in the final product at market level.

Few data are available on veterinary product use in Lebanon, and it is not enough to cover the needs of an official residue control plan.

Chapter 6: Aim, planning and results of the PhD thesis

6.1 OBJECTIVES OF THE STUDY

Based on country-specific estimates per capita milk consumption classifications, Lebanon is categorized to be among the countries which have a high intake of milk defined as per capita milk consumption/year of >150 kg (FAO, 2008 Adapted from IFCN Dairy Report 2006, Chapter 3.6). Moreover, IFAD estimates that the local dairy products consumption in Lebanon per capita is 114 litres (IFAD, 2017). Furthermore, dairy industries do not control effectively antibiotic residues, only industries abiding by the requirements of the General Principles of Food Hygiene perform rapid tests that detect beta-lactams and tetracycline residues in raw milk. Veterinary antibiotics are sold very easily without any prescription to any citizen. Decisions concerning veterinary prescriptions exist only on papers but they are not applied in field.

The Lebanese dairy farms endorse around 81626 dairy cows (FAOSTAT, 2017) that are distributed over small, medium and large dairy farms raised in a semi-intensive and intensive breeding system. Economical issues play an important role in the Lebanese farms creating a lack of hygiene leading to an imprudent antibiotic use which promotes the affection of milk quality knowing that none of the dairy farms check for antibiotic residues in their produced milk. Unfortunately, no official control plan on antibiotic residues in raw milk is established in order to prevent milk contamination through veterinary antibiotics. Actually, requirements for veterinary medicines in Lebanon are not established properly, and

few epidemiological studies on the occurrence of drug residues in raw milk are performed.

Training activities concerning controlling antibiotic residues in milk, were performed in Sardinia-Italy for a period of 2 months in the biggest two cheese making plants. Moreover, trainings were performed as well in two accredited laboratories in order to understand the difference between self-checks and official control on veterinary residues. As a result, we decided to find out the prevalence and identify antibiotic residues in dairy cow's bulk tank milk produced in Lebanon.

As a first step, a questionnaire formed of 43 questions was established to evaluate the knowledge level of 100 Lebanese farmers randomly chosen. The questionnaire covered different subjects related to the farmer's knowledge level and antibiotic usage. Furthermore, 1020 dairy raw milk samples were collected from the 7 Lebanese Governorates over 10 months starting from March 2018 until December 2018. The sampling epidemiology was decided based on the medium and large dairy farms distribution in the 7 Lebanese Governorates. Samples were checked for inhibitors using microbiological inhibitor tests. All double positive tested samples were checked for penicillin G, tetracycline and florfenicol residues using high-performance liquid chromatography (HPLC) test. One hundred positive tests found using HPLC-DAD were tested using immuno-chromatographic antibiotic residues rapid test.

The objective of this thesis is to perform an epidemiological study on the level of raw milk contamination by antibiotic residues in all Lebanese Governorates, define the knowledge level of dairy farmers that were selected objectively to represent all Lebanese dairy farmers and increase the awareness level against antibiotic residues in dairy raw milk.

6.2 MATERIALS AND METHODS

6.2.1 Study area

Lebanon with an area of 10,452 Km2, is located in Western Asia, Eastern part of the Mediterranean area and approximately at 35˚N, 35˚E. Form a sky view, Lebanon has approximately a rectangular shape that becomes narrower to the North and the South. The widest point is 88 km and its narrowest is 32 km. Lebanon Weather has four seasons: Winter, Spring, Summer and Autumn. Lebanon is formed of eight Governorates, Beirut, Mount Lebanon, the North, the South, Baalbek-Hermel, Beqaa Valley, Nabatieh and Akkar. Beirut, the Lebanese capital, is the only Governorate that does not endorse dairy farms. Dairy cattle in medium and large farms that mainly covers the Lebanese dairy market demands, are mostly distributed within the Beqaa Valley then in Mount Lebanon, followed by Baalbek-Hermel, Nabatieh, Akkar, the South and lowest number is found in the North.

6.2.2 Study design

The medium (10 to 40 cows) and large farms (more than 40 cows) in Lebanon, produce milk intensively with professionalism when compared to the small farms (less than 10 cows). The milk produced is directly sold to the big milk factories and when talking about the biggest farms, the owner has his own dairy plant.

The small producers can be mainly described as unprofessional breeders. They produce a small quantity of milk that is not enough to be sold directly to the milk factories. This is why collection centers are collecting the milk from small farms and mix it all together in refrigerated tanks, increasing the risk of milk cross-contamination with residues. The animals are few but the number of small farms is

higher than the total of medium and large farms. They represent around 40-50% of dairy cow's population in Lebanon. Knowing that the collection centers are mixing all milk together, the percentage of dilution for any antibiotic residue is very high and may interfere with the sensitivity of the microbiological and rapid tests as well as the homogeneity of the results.

In developing an epidemiological study on antibiotic residues in milk produced in Lebanon, sampling was performed based on the three different situations:

- a sampling of the bulk tank milk produced in the medium and big farms that are more than 12.000 farms was provided, to be representative of the farms that are more developed playing a more important role economically;

- a sampling from the small farms that are more than 15.000, is provided as well;

- and despite the lacking of sensitivity as an effect of the residue dilution that occurs while mixing contaminated milk with milk of different farms, a sampling at the collection points is provided as well that covers a big number of mixed milk from small farms.

6.3 EPIDEMIOLOGIC STUDY

The questionnaire survey was established in order to determine the awareness and knowledge level concerning the prudent use of antibiotics in dairy farms. Questions were built based on a personal experience with small, medium and large farm owners. Some of them are similar but asked in tricky way using different approaches, taking into consideration that farmers are not familiar with this activity. The questionnaire consists of 42 questions that treat different topics that are general

information about the farmer's knowledge; farmer's knowledge about antibiotics; the main three antibiotics used in the selected farms; the farmer's access to antibiotics and farmer's knowledge about antibiotic usage and the methods of using it. One hundred selected farms were chosen randomly and in an epidemic distribution that represent the medium and large farms in the 7 Lebanese Governorates. The majority of farmers, located in rural areas, are under-educated but very few were understanding questions immediately; this is why the questionnaire survey was explained in the Lebanese Arabic language, face to face in details to the selected farmers, in order to prevent any misunderstanding of questions. To make it easier to the farmers, all questions were close ended.

The questionnaire covered several subjects as described in the Annex 1 of the thesis.

6.3.1 **Results and interpretation of the questionnaire**

Descriptive analysis was done for the results using the statistical software SPSS v16.

- General information concerning the farmer and his farm.

Results concerning the general information about farmers and his are shown in table 5 below:

Table 5: General information concerning the farmer and the farm

Questions	Results	
1. Are you the farm owner?	Yes **85%**	No **15%**
2. What do you do with the animal if the treatment failed?	Sell **100%**	
3. What do you do with milk during antibiotic treatment?	Throw **79%**	Sell **21%**
4. What does the milk with antibiotics used for?	Not used **79%**	Dairy products **21%**
5. When using antibiotics that is excreted in milk production do you usually throw the milk?	Yes **83%**	No **17%**
6. What do you care more about when using antibiotic that is excreted in milk, selling the milk or human health?	Selling the milk **14%**	Human health **86%**
7. How much do you sell 1L of milk containing antibiotics?	No answers	
8. Do you know the quantity of AB used in your farm per year? If yes, how many?	No **100%**	

Eighty-five percent of the questioned people were the farm owners and 15% were workers at the farm. None of the questioned people knew about the quantity of antibiotics used yearly in their farms. When treatment protocol fails to treat the animal, the latter is sold for meat production. Eighty-three percent of the questioned people throw the contaminated milk when the animal is treated with antibiotic, but 17% don't. However, when they are asked indirectly about the milk produced when the animal is treated, 79% of them discard contaminated milk and 21% sell it. 79% of the questioned people believe that milk contaminated with antibiotic residues is not used for dairy production but 21% of them believe that it is used. Concerning human health, 86% of the questioned are more interested than 16% who want to sell the milk at any price regardless of the outcome.

- Farmer knowledge about antibiotics

Results concerning the general information about farmer knowledge about antibiotics are shown in table 6 below:

Table 6: Farmer knowledge about antibiotics

Questions	Results		
1. Do you know what an antibiotic is?	Yes **92%**	No **8%**	
2. What are antibiotics used for?	Viral & bacterial infections **53%**	Bacterial infection **40%**	Viral infection **7%**
3. Can antibiotics cure bacterial infections?	Yes **100%**		
4. Can antibiotic cure viral infections?	Yes **58%**	No **42%**	
5. Do you think the use of antibiotics will speed up the recovery of any illness in cows?	Yes **58%**	No **42%**	
6. Do you think frequent use of antibiotics will decrease the efficacy of treatment when using the antibiotic again?	Yes **31%**	No **69%**	
7. Have you heard about antibiotics resistance?	Yes **31%**	No **69%**	
8. Is the efficacy better if the antib newer and cost more?	Yes **81%**	No **19%**	
9. Do you know about agonist and antibiotics?	No **100%**		
10. Have you ever seen any adverse reaction when you were using antibiotics?	Yes **19%**	No **81%**	
11. What is (are) the common adverse reaction(s) of antibiotics?	Not educated **81%**	Educated **19%**	
12. What do you do for the adverse reactions?	Professional recommendations **81%**	Unprofessional recommendations **19%**	
13. Do you think you can treat common infectious diseases with antibiotics successfully by yourself?	Yes **21%**	Not sure **79%**	

Only 8% of the farmers do not know what an antibiotic is, but 92% of them answer that they do know. However, when asking them about antibiotic usage, 53% believe that it can cure viral and bacterial infections, 40% said that it can cure bacterial infections only and 7% only for viral infection. Whereas when asking them indirectly if antibiotic can cure bacterial infections all of the questioned people agreed, but when talking about treating viral infections, only 58% of them agreed and 42% disagreed. Only 31% believe that frequent use of antibiotics will decrease the efficacy of treatment when using again the same antibiotic but 69% of them disagreed. 58% of the questioned believe that antibiotics will speed up the recovery of any illness in cows and 42% disagree. When talking about antibiotic resistance, only 31% of the questioned people knew about it but 69% didn't.

Moreover, none of the questioned people gave us a price for contaminated milk sold in the market. 81% of the questioned believe that newer and more expensive antibiotics are more effective, but 19% of them think that it is not true. None of the questioned farmers knows about agonist and antagonist antibiotics. Only 19% of the questioned have experienced adverse drug reaction and 81% have never seen it, but when I asked them about the symptoms of an adverse reaction, 81% of them had no idea and 19% replied correctly. 81% of the questioned farmers ask for professional recommendation in case of an adverse reaction but 19% ask any person that not necessary a veterinarian. From all the questioned people 21% believes that they can cure a common infectious disease using antibiotics by themselves but 79% aren't certain.

- **Main three antibiotics used at farms:**

Results concerning the main three antibiotics used at farms are shown in table 7 below:

Table 7: Main three antibiotics used at farms

Questions	Results		
What are the main three antibiotics that you use in your farm?	Penicillin 37%	Florfenicol 34%	Tetracycline 25%

The main three antibiotic used in the 100 farms where the questionnaire was performed are, penicillin (37%), florfenicol (34%) and tetracycline (25%).

- **Access to antibiotics:**

Results concerning access to antibiotics are shown in table 8 below:

Table 8: Access to antibiotics

Questions	Results			
1. How difficult is your access to antibiotics?	Very easy **100%**			
2. From where do you buy antibiotics that are required by a veterinary prescription?	Veterinary company/pharmacy or veterinarian **80%**	Veterinarian **11%**	Veterinary company **5%**	Veterinary pharmacy **4%**
3. From where do you buy antibiotics for self-medication?	Veterinary company/pharmacy or veterinarian **80%**	Veterinarian **11%**	Veterinary company **5%**	Veterinary pharmacy **4%**

All questioned people have a very easy access to buy antibiotics in order to perform a treatment whether prescribed from a veterinarian or not. 80% of them buy their antibiotics form a veterinary company/pharmacy or a veterinarian, 11% of them buy antibiotics only from veterinarians, 5% only from veterinary companies and 4% only from veterinary pharmacies.

- **Information about antibiotic usage:**

Results concerning information about antibiotic usage are shown in table 9 below:

Table 9: Information about antibiotic usage:

Questions	Results			
1. What is your information source of antibiotics usage?	Professional recommendation **90%**		Unprofessional recommendation **10%**	
2. How do you find your access to your source of information?	Veterinary company/pharmacy or veterinarian **80%**	Veterinarian **11%**	Veterinary company **5%**	Veterinary pharmacy **4%**

Ninety percent of the questioned people have a professional recommendation for antibiotic usage and only 10% rely on unprofessional recommendations. However, when asking about the source of information, 80% rely on a veterinary company/pharmacy or a veterinarian, 11% on veterinarians only, 5% on veterinary companies and 4% only on veterinary pharmacies.

- **Method of antibiotic usage**

Results concerning method of antibiotic usage are shown in table 10 below:

Table 10: Method of antibiotic usage:

Questions	Results			
1. Have you ever used antibiotics without consulting a veterinarian?	Yes **100%**			
2. What is the reason of using antibiotics without consulting a veterinarian?	Experience **68%**	Economical **32%**		
3. What was your selection of antibiotic based on?	Previous doctor's prescription **59%**	His own experience **41%**		
4. What did you consider when selecting antibiotics?	Antibiotic brand **7%**	Indication of use **65%**	Price of the antibiotics **12%**	Type of the antibiotic **16%**
5. Did you ever checked the instruction leaflet inside the antibiotic package in case of self-treatment?	Never **68%**	Sometimes **32%**		
6. How much did you understand the instructions on the leaflet?	Do not understand at all **79%**	Partly understood **21%**		
7. How did you know the dosage of antibiotics?	Experience from previous usage **67%**	By consulting a veterinarian **33%**		
8. Did you change the dosage of antibiotics deliberately during the course of self-treatment?	Yes **45%**	Sometimes **55%**		
9. Why do you switch antibiotics during the course of self-treatment?	Animal is not getting better **91%**	Economical **9%**		

10. Did you ever switch antibiotics during the course of self-treatment?	Yes, sometimes **100%**				
11. Why do you switch antibiotics during the course of self-treatment?	No effect **93%**		Economical **4%**		The antibiotic runs out **3%**
12. How many different antibiotics did you use maximally during a single illness?	Two **83%**		Three **17%**		
13. Have you ever found that you had used the same antibiotics with different commercial names during the same treatment course?	Yes **95%**		No **5%**		
14. Do you usually choose an antibiotic that is not excreted in milk production?	No **88%**		Yes **12%**		
15. When do you normally stop using antibiotics?	After symptoms disappeared **63%**	After professional recommendation **14%**	After a few days regardless of the outcome **12%**	A few days after the recovery **7%**	After antibiotics ran out **4%**

In all visited farms, antibiotics were used without veterinary consultation. The reason was due to the experience of 68% of the farmers that they think it is enough to treat the animal by themselves and 32% of the questioned had some economical issues.

In the case of self-treatment, the selection of antibiotics was based on the previous doctor's prescriptions in 595 of the cases and 41% on the farmer's experience. 65% of the farmers consider the indication of the antibiotic usage when choosing an antibiotic, 16% decide based on the type of the antibiotic, 12% based on the price of

the antibiotic, and 75 of the antibiotic brand. Only 32% of the questioned check sometimes the antibiotic leaflet but 68% of the questioned have never checked it. The leaflet was partially understood by 21% of the farmers but 67% of them did not understand it at all. 33% of the questioned farmers ask the veterinarian about antibiotic dosage but 67% rely on previous usage. 45% of the farmers change antibiotic dosage deliberately during a treatment and 55% do it sometimes. 91% of them accuse this change by the animal that is not becoming better but 9% have some economical issues. All of the questioned farmers have changed antibiotics during a course of deliberate treatment. 93% of them did it because they saw no better effect on the animal case, 4% have economical issues and 3% finished the antibiotic bottle during the treatment and decided to use another antibiotic to avoid buying a new bottle.

Eighty-three percent of the selected farmers can switch two types of antibiotics during a treatment and 17% switch three types. Moreover, 95% of the farmers have used the same antibiotic molecule with different commercial names but 5% did not. Antibiotics excreted in milk are only chosen by 12% of the farmers but 88% didn't rely on this criterion to choose their antibiotic for treatment. 63% of the farmers stop the treatment when symptoms disappear, 14% after professional recommendations, 12% after few days regardless of the outcome, 7% a few days after the recovery of the treated animal and 4% when the antibiotic ran out.

6.4 SAMPLING PLAN, MILK SAMPLE COLLECTION AND TRANSPORTATION

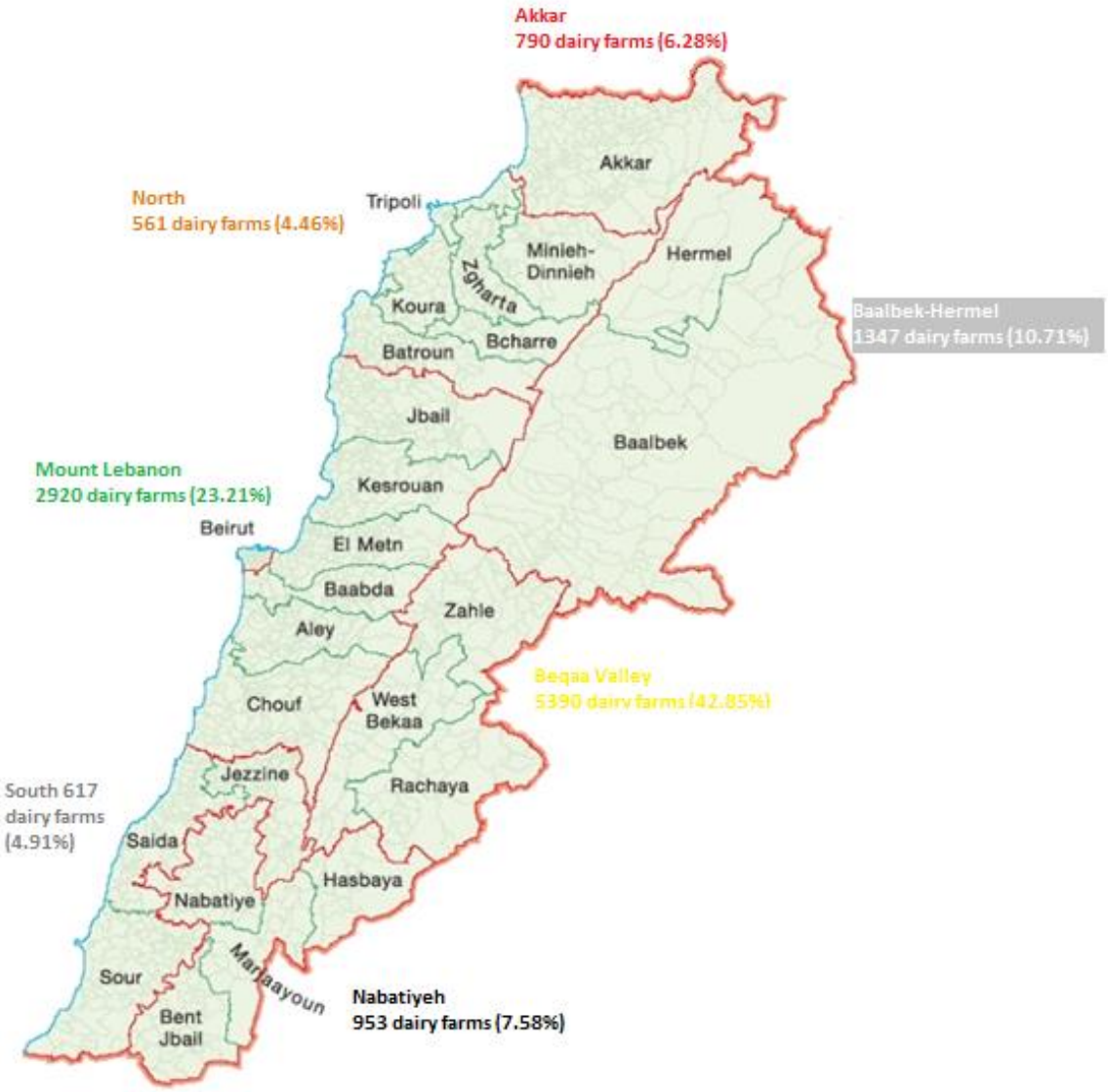


Figure 1: Lebanon Map representing 7 Governorates from where 1020 raw milk samples were collected based on the epidemiological distribution of medium and large dairy farms

Governorates names and number of medium-large farms is coloured as the colour coding of the survey.

Table 11: Showing the number of samples collected per month, the approximate number of medium and large dairy farms in Lebanon and the colour code of each Governorate samples

	Beqaa Valley	Mount Lebanon	Baalbek-Hermel	Nabatiyeh	Akkar	South	North	Total
Number of samples/month	42	24	11	8	7	5	5	102
1Population percentage of medium and large farms in Lebanon	42.85	23.21	10.71	7.58	6.28	4.91	4.46	100
Approximate number of medium and large farms in the Governorate	5390	2920	1347	953	790	617	561	12578
Colour code of the Governorate sample	yellow	green	White	black	Red	transparent	Gold	7 different colours

The medium to large dairy farms are distributed on seven governorates (figure 1; table 11). The Bekaa Valley enclosing the largest number of farms that is around (5390) 42.85% of medium to large dairy farms. In Mount Lebanon, almost (2920) 23.21% of medium to large dairy farms are distributed on different altitudes in different villages. Baalbek-Hermel, contains (1347) 10.71% of those farms size. Nabatiyeh covers (953) 7.58%, Akkar, the South and the North of Lebanon covers (790) 6.25%, (617) 4.91% and (561) 4.46% respectively. The approximate total number of medium to large farms represented is 12578 farms.

Based on the approximated number of medium and large dairy farms distribution in Lebanon, that was collected from the Ministry of Agriculture, number of samples to be collected per month was decided. Beqaa Valley medium and large dairy farms were represented by 42 samples, Mount Lebanon was represented by 24 samples, Baalbek-Hermel by 11 samples, Nabatiyeh, Akkar, the North and the South were represented by 8, 7, 5 and 5 samples respectively. The total number of samples collected per month was 102 raw milk samples. Over the whole study, 1020 raw milk samples were collected.

Moreover, to prevent any mistake related to samples, sterile bottles cap used to collect samples from different governorates were colour coded. Beqaa Valley was represented with yellow cap colour, Mount Lebanon with green cap colour, Baalbek-Hermel with white, Nabatiyeh, Akkar, the North and the South were represented with black, red, gold and transparent cap colours respectively.

Figure 2: Showing raw milk samples bottles colour coded inside the freezer regulated at -20℃.

Figure 3: Showing 30 ml raw milk samples separated and color coded. The red cross sign on the top of each sample means that it was found positive for microbiological inhibitor test.

6.4.1 **Sampling plan**

A specific plan is used to collect samples from farm bulk milk in a proportional way in all governorates based on the distribution of medium and large farms in Lebanon. The basic path is determined by the medium and large farms in each governorate. Samples from small farms were collected randomly depending on the pathway during the day. Each month, 102 samples were collected for a period of 10 months starting from March 2018 until December 2018.

The collection technique is a critical point; it depends from the tank size and model. First, we had to check the temperature and the pH of milk that are very important to verify if the milk was stored in proper conditions or not so we can prevent any non-compliancy that might affect the results of the tests.

6.4.2 **Sampling collection**

When a dripper was available, it was used to collect the samples in the sterile color coded bottle.

Bulk tanks can be associated or not to an automatic agitator. Based on the criteria of the bulk tank, manual sampling protocol was selected. Moreover, a specific manual mixer made from stainless steel was used with a cup from the same quality. The dimension of the mixer and the cup are given by UNI (Ente Nazionale Italiano di Unificazione; Via Battistotti sassi, 11B 20133 Milano, Italia). A specific regulation was made to determine the specific sizes of the used mixer to collect manually from bulk milk tanks. We mixed the milk for 5 minutes (if manually) or 1 minute (if there is an automatic agitator associated to the milk tank), then collected the sample. The quantity and number of samples collected are based on the European norm in 'UNI EN ISO 707' on December 2008. For instance, if many tanks were present in the same farm, samples from each tank were collected than mixed all together and one sample from this mixture was collected. For a 1000 liters tank, five samples should be collected, mixed, then one sample is collected from the mixture. The materials used for collection should be cleaned with a detergent then properly washed with water and dried before collecting another sample in order to prevent a false positive result when using inhibitor test.

6.4.3 **Labelling and transportation**

Samples collected were labeled with the farm or the owner name and the date of collection. The colored cap of each bottle was used to identify each Governorate. After collecting and labeling the samples, they were stored immediately in a 12 Volts fridge controlled on a temperature of 4°C. Samples were labeled and transported based

on the methods described by ISO 707:2008 (ISO, 2008). Collected samples were tested not more than 24 hours post-collection using a commercial Microbiological Inhibitors Test (MIT), for the detection of antibiotic residues in milk at the Lebanese University Veterinary Medicine Department. In some cases, few samples were tested 48 hours post-collection. The milk sample was 300 ml. This quantity was enough to perform MIT, Charm test as a rapid test and High Performance Liquid Chromatography test (HPLC) as a chemical test. The 300 ml samples were divided in 2 samples of 30 ml that were used for MIT and 270 ml that was frozen on -20°C, to be used later for the rapid and chemical test.

6.5 INHIBITORS MICROBIOLOGICAL DETECTION TEST

6.5.1 Material and methods

Inhibitor microbiological detection test was performed as a first screening test in this PhD thesis. One thousand and three hundred MIT (Delvotest® T, DSM, Netherland). Tests were received refrigerated. During the testing period, MIT kits were stored upright in their original packaging in a refrigerator at a temperature between 4-8°C.

Delvotest® is a standard diffusion test that is used to detect residues in raw milk derived from antibotics and sulphonamides used in farms by veterinarians and farmers. The test is formed of small wells containing a solid agar medium seeded, a standardized number of *Bacillus stearothermophilus* var. *calidolactis* spores with necessary nutrients for growth purposes and an antifolate trimethoprim (DSM, 2011). The medium color is violet because of the pH indicator bromocresol purple. The quantity of milk needed to perform one test is 0.1 ml and the dry incubation period is 3 hours +/- 15 mins at 64°C +/- 2°C. When milk samples free of antibiotics or

contaminated with antibiotic residues below the sensitivity detection level of MIT are added to the well and incubated, the bacteria can grow and germinate. This bacterial growth will lead to a change in the indicator color that becomes yellow. When a milk sample contaminated with antibiotic residues that is equal or exceed the sensitivity of the test, bacterial growth is inhibited leading to the persistence of purple color (DSM, 2011).

6.5.2 **MIT sensitivity**

Table 12 represents the sensitivity of MIT for the most used antibiotics in the world (not all inhibitors are included knowing that the test is sensitive to many more antibiotic drugs and sulfonamides). The sensitivity corresponds to the concentration for which 95% of the samples analyzed are positive. The best test sensitivity is reached when using the required time (3 hours ± 5 mins). To double check the required time, a negative control test should be mixed with a blank milk sample that will be incubated until the color of the agar inside the well becomes yellow (DSM, 2011).

Table 12: Sensitivity of MIT for the most used antibiotics (DSM, 2011)

Antibiotic Class	Antibiotic	MRL	Ampoule CCB (ppb)
Penicillins	Amoxicillin	4	4
	Ampicillin	4	4
	Penicillin G	4	2
	Cloxacillin	30	6
	Oxacillin	30	30
Tetracyclines	Oxytetracycline	100	100
	Chlortetracycline	100	150
	Tetracycline	100	70
	Doxycycline	0	50

Sulfonamides	Sulfamethazine	100	135
	Sulfathiazole	100	40
	Sulfadimethoxine	100	40
	Sulfadiazine	100	40
Macrolides	Tilimicosin	50	60
	Tylosin	50	35
	Erythromycin	40	160
Aminoglycosides	Neomycin	1500	60
	Gentamycin	100	65
	Kanamycin	150	1010
	DH/Streptomycin	200	4240
	Spectinomycin	200	2010
Cephalosporins	Cephapirin	60	6
	Ceftiofur (pur)*	100	20
	Cefoperazone	50	40
	Cephalexin	100	30
	Cefquinome	20	40
Others	Lincomycin	150	220
	Chloramphenicol	0	4100
	Trimethoprim	50	110
	Rifamixin	60	40
	Dapson	0	30

*Ceftiofur with metabolites has a detection limit about 4 times higher.

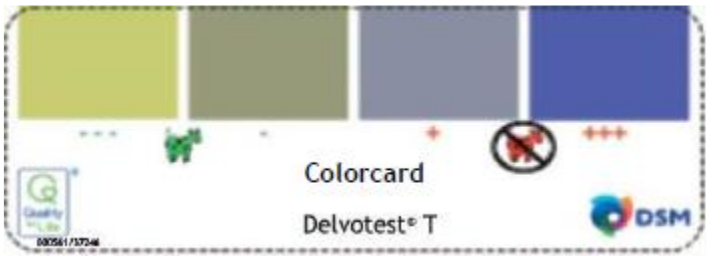
MIT was performed on collected raw milk samples following the procedures provided by producer at the Lebanese University of Agriculture and Veterinary Medicine Laboratory. Milk samples were tested at the same day. If not, samples were

stored at the refrigerator at a temperature of 4℃ and tested after a maximum of 48 hours of the sampling activity.

Results were read based on the MIT colorcard that is found in every box.

Figure 4: Showing the reading colors of MIT based on the colorcard

Figure 5: Showing the colorcard of the MIT



All positive results were double checked using the same MIT tests to have a double confirmation of non-compliant tests.

6.5.3 Inhibitors microbiological detection test results

Table 13: Showing results of the 1020 milk samples collected from seven governorates in Lebanon and tested using MIT

Month/Governorate	Mount Lebanon n=24	Beqaa Valley n=42	Baalbek/ Hermel n=11	North n=5	South n=5	Nabatiyeh n=8	Akkar n=7	%
March	**5**	**6**	0	0	0	**1**	0	**11.7647**
April	**7**	**14**	0	0	0	0	0	**20.5882**
May	**2**	**7**	**2**	**1**	0	**1**	0	**12.7451**
June	**3**	**4**	0	**2**	0	**1**	**6**	**15.6863**
July	0	**8**	0	**1**	0	**1**	**7**	**16.6667**
August	**5**	**10**	**3**	0	0	**1**	0	**18.6275**
September	**6**	**9**	**8**	**1**	0	**2**	0	**25.4902**

October	**5**	**7**	**8**	**3**	0	**3**	0	**25.4902**
November	**4**	**16**	**7**	**3**	**3**	**2**	0	**34.3137**
December	**2**	**18**	**9**	**1**	**1**	**4**	0	**34.3137**
Total number of samples per Governorate	240	420	110	50	50	80	70	
Total numbers of positive samples per Governorate	**39**	**99**	**37**	**12**	**4**	**16**	**13**	
Total number of samples in this study	**1020**							
Total number of positive samples in this study	**220**							
% of positive samples per governorate	**16.575**	**24.043**	**34.309**	**24.48**	**8.16**	**20.4**	**18.94**	

6.5.4 **Interpretation**

One thousand twenty dairy raw milk samples were collected from seven Lebanese Governorates over 10 months starting from March 2018 until December 2018 (table 13). Each month one hundred and two collected raw dairy milk samples were tested for the presence of the inhibitors using a microbiological test MIT. Results show that 220 (22 %) out of 1020 randomly collected dairy raw milk samples were found to be double positive for the presence of inhibitors above the MRL.

When calculating results based on the number of contaminated samples in each Governorate in every month starting from March 2018 until December 2018, data shows that; in March 2018, 6 out of 42 collected samples were found contaminated with inhibitors in the Beqaa Valley, 5 out of 24 in Mount Lebanon and 1 out of 8 in Nabatiyeh. In Baalbek-Hermel, the north, The South and Akkar, no positive sample were found.

In April 2018, 14 out of 42 samples were found to be contaminated with inhibitors in the Beqaa Valley and 7 out of 24 samples in Mount Lebanon. No other samples were found contaminated with inhibitors in Baalbek-Hermel, The North, The South, Nabatiyeh and Akkar.

In May 2018, 7 out of 42 samples were found positive for inhibitors in Beqaa Valley, 2 out of 24 in Mount Lebanon, 2 out of 11 in Baalbek-Hermel, 1 out of 8 in Nabatiyeh and 1 out of 5 in the North. No other samples were found positive in the South and Akkar.

In June 2018, 6 out of 7 samples were found positive for inhibitors in Akkar, 4 out of 42 in Beqaa Valley, 3 out of 24 in Mount Lebanon, 2 out of 5 in the North and 1 out of 8 in Nabatiyeh. No positive results were found in Baalbek-Hermel and the South.

In July 2018, 8 out of 42 samples were found positive in the Beqaa Valley, all collected samples from Akkar were positive, 1 out of 5 in the North and 1 out of 8 in Nabatiyeh. No positive results were found in the South and Baalbek-Hermel.

In August 2018, 10 out of 42 samples were found positive in the Beqaa Valley, 5 out of 24 in Mount Lebanon, 3 out of 11 in Baalbek-Hermel and 1 out of 8 in Nabatiyeh. No other samples were found contaminated in the North, the South and in Akkar. In September 2018, 9 out of 42 results were contaminated with inhibitors in the Beqaa Valley, 8 out of 11 in Baalbek-Hermel, 6 out of 24 in Mount Lebanon, 2 out of 8 in Nabatiyeh and 1 out of 5 in the North. No other samples were contaminated in the South and Akkar.

In October 2018, 8 out of 11 samples were found contaminated with inhibitors in the Baalbek-Hermel, 7 out of 42 in the Beqaa Valley, 5 out of 24 in Mount Lebanon, 3 out of 5 in the North and 3 out of 8 in Nabatiyeh. No positive results were found

contaminated in the South and Akkar.

In November 2018, 16 out of 42 samples were found positive in the Beqaa Valley, 7 out of 11 in Baalbek-Hermel, 4 out of 24 in Mount Lebanon, 3 out of 5 in the North and the South, 2 out of 8 in Nabatiyeh. no positive results were found in Akkar.

In December 2018, 18 out of 42 samples were found positive in the Beqaa Valley, 9 out of 11 in Baalbek-Hermel, 4 out of 8 in Nabatiyeh, 2 out of 24 in Mount Lebanon, and 1 out of 5 in the North and the South. No contaminated samples were found in Akkar.

When calculating results based on the number of samples that were found contaminated each month starting from March 2018 until December 2018, data revealed the following;

in March 2018, 12 (11.7647 %) out of 102 collected dairy raw milk samples were found positive for inhibitors using MIT in the 7 Governorates. In April 2018, 21 (20.5882 %) out of 102 samples were found positive using MIT. In May 2018, 13 (12.7451 %) out of 102 samples were found positive. In June 2018, 16 (15.6863 %) out of 102 samples were found positive. In July, 17 (16.6667 %) out of 102 samples were found positive. In August 2018, 19 (18.6275 %) out of 102 samples were found positive. In September 2018, 26 (25.4902 %) out of 102 samples were found positive. In October 2018, 26 (25.4602 %) out of 102 samples were found positive. In November and December 2018, 35 (34.3137 %) out of 102 samples were found positive.

When analyzing the results based on the total collected samples per month from March 2018 until December 2018 in each Governorate, results show that in Baalbek-Hermel Governorate, 37 (34.309%) out of 110 collected dairy raw milk samples were found

non-compliant based on the MIT test. At the North, 12 (24.48 %) out of 50 collected samples were found contaminated with inhibitors when tested with MIT. In Beqaa Valley, 99 (24.043 %) out of 420 collected samples were found contaminated with inhibitors. In Nabatiyeh, 16 (20.4 %) out of 80 collected samples were found contaminated with inhibitors. 13 (18.94 %) out of 70 samples were found contaminated with inhibitors in Akkar. In Mount Lebanon, 39 (16.575 %) out of 240 samples were found contaminated with inhibitors. In the South 4 (8.16 %) out of 50 samples were found contaminated with inhibitors.

When classifying the 7 Lebanese Governorates based on the percentage of dairy raw milk contamination by inhibitors from March 2018 until December 2018, Baalbek-Hermel states in the highest risk with a percentage of 34.309 % of contaminated milk. In the second place, the North have 24.48% of his produced dairy milk contaminated with inhibitors. The 3rd place goes for Bekaa Valley which is the biggest dairy milk producer in Lebanon with a percentage of 24.043% of contaminated milk with inhibitors. Nabatiyeh is classified at the fourth place with a percentage of 20.4% of contaminated milk. Akkar, Mount Lebanon and the South, are classified with fifth, sixth and seventh respectively with a percentage of 18.94%, 16.575% and 8.16% of milk contaminated with inhibitors.

6.6 IMMUNO-CHROMATOGRAPHIC ANTIBIOTIC RESIDUES RAPID TEST

6.6.1 Material and methods

Two hundred and twenty contaminated samples with inhibitors were detected using the microbiological test MIT. The inhibitors contaminating raw milk samples might be antibiotic residues or any bacterial growth inhibitor such as detergent. In

order to identify if the inhibitors are veterinary drug residues, immune-chromatographic antibiotic residues rapid test is used.

Based on the questionnaire answer related to the main tree antibiotic used in the Lebanese dairy farms, immune-chromatographic antibiotic residues rapid tests that can detect penicillin, florfenicol and tetracycline at the maximum residue level were used. The Charm test is an immune-receptor assay utilizing ROSA® (Rapid One Step Assay) lateral flow technology (Charm Sciences, 2016).

One hundred of each Charm® TRIO (CT) and Charm® Amphenicol (CA) tests were shipped from Italy to Lebanon and used as recommended by the manufacturer to detect antibiotic residues in dairy raw milk samples. The number of tests were not enough to check all positive samples for inhibitors, but sufficient to compare microbiological antibiotic residues test to the immuno-chromatographic antibiotic residues rapid test. This is why, this Immuno-chromatographic antibiotic residues rapid test was performed after confirming the positive results by the High-performance liquid chromatography (HPLC) test.

Based on the questionnaire answers about main antibiotic usage, two tests CT and CA, were used to cover the molecules that can mainly be present in the 220 raw milk samples that were found double positive using Devotest®. The CT test can detect beta-lactams, sulfa drugs and tetracyclines and the CA test detects the family of amphenicol metabolites (Charm Sciences, 2016).

Amphenicol, beta-lactams, sulfa drugs and tetracyclines drugs interact with colored beads in the lateral flow test strip and the color intensity in the test and control zones can be read visually (Charm Sciences, 2016).

6.6.2 **Sensitivity level of the CA and CT tests**

CA test sensitivity can be specific with a percentage of 99% for amphenicol-free raw milk samples. The detection level of chloramphenicol, is 0.10 to 0.15 ppb which is at or below the EU MRPL (Minimum Required Performance Level), as for florfenicol and thiamphenicol it is 4 to 6 ppb.

CT test sensitivity can be specific with a percentage of 90% for beta-lactam, sulfa drugs and tetracycline free raw milk samples with 95% confidence. In table 14, the sensitivity level and selectivity of CT test are shown (Charm Sciences, 2016).

Table 14: Show sensitivity and selectivity of CT test

Drug family	Drug molecule	Detection level (ppb)
Beta-lactam	Amoxicillin	3.5
	Ampicillin	8.8
	Ceftiofur & metabolites	50
	Cephapirin	14.5
	Cloxacillin	8.5
	Penicillin G	2.0
Sulfa drugs	Sulfadimethoxine	7.6
	Sulfamethazine	9.2
Tetracycline drugs	Chlortetracycline	34
	Oxytetracycline	53
	Tetracycline	42

When test strips are received, the desiccant was inspected for its blue color in order to make sure that they are still valid or not. Before starting to analyze milk samples, positive and negative control samples were tested as required by the manufacturer before using the kit (Charm Sciences, 2016).

6.6.3 **Method of using CA and CT Tests**

Milk samples were shacked before using to make sure that the sample is homogeneous. Strips were labeled to prevent false readings. Before removing the sealing of the strip, it was placed inside the incubator. After removing the sealing to

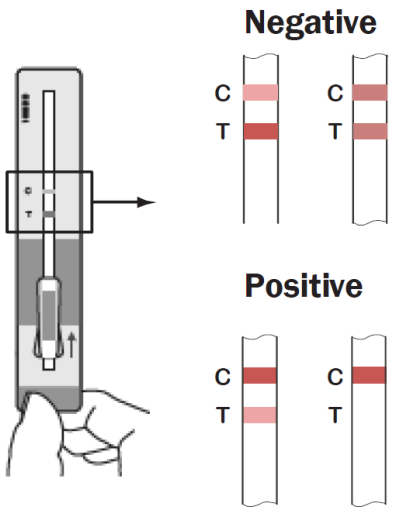
the limit line allowed, 300 µl of raw milk was dropped slowly using a micropipette avoiding foam and bubbles.

For CA, the strip was incubated for a period of 8 minutes on a temperature of 40°C. For CT test strip, the strip was incubated for a period of 3 minutes on a temperature of 56 °C (Charm Sciences, 2016).

Results are read in a maximum of 1 minutes after making sure that the control line is clear. In order to prevent any false results, we considered that the negative results for antibiotic residues are when the line referring to the antibiotic is darker or the same color as the control line and the positive results are confirmed when the line referring to the antibiotic is complete and lighter than the control line (Charm Sciences, 2016).

Out of 100 tests of each package (CT and CA) 4 strips were used to check positive and negative control of the tests and one strips was used to recheck an uncertain result.

Figure 6: Showing the visual reading of For Charm® tests.



6.6.4 Result of the immuno-chromatographic antibiotic residues rapid test

Table 15: Representing results of the 95 CA and TRIO test results performed on 95 positive samples found using MIT and HPLC-DAD tests

Criteria and Antibiotic	Results		Total number of tests	Percentage of positive samples
Positive	92		95	96.8
Negative	3		95	3.1
Antibiotic	**Positive**	**Negative**		
Amphenicol	38	57	95	40.0
Penicillin G	59	36	95	62.1
Tetracycline	41	54	95	43.2
Sulfa	31	64	95	32.6
Penicillin G/Tetracycline/Sulfa	5		95	5.3
Pen/Tetracycline	15		95	15.8
Tetracycline/ Sulfa	10		95	10.5

6.6.5 Interpretation

The immuno-chromatographic antibiotic residues rapid test, Charm® test, detects antibiotic residues that are higher than the maximum residue limits. Nighty five double positive raw milk samples were chosen arbitrary out of 220 double positive samples tested with MIT (table 15). Out of 95 raw milk samples, 92 (96.8%) samples were found contaminated with antibiotic residues with a percentage above the European maximum residue limit.

Out of 95 raw milk samples, 38 (40%) dairy raw milk samples were found contaminated with amphenicols; fifty-nine (62.1%) raw milk samples were found contaminated with penicillin G; forty-one (43.2%) raw milk samples were found

contaminated with tetracycline; thirty-one (32.6%) raw milk samples were found contaminated with sulfa drugs.

Moreover, five (5.3%) raw milk samples were found contaminated with Penicillin G, tetracycline and sulfa residues; fifteen (15.8%) raw milk samples were found contaminated with penicillin G and tetracycline drug residues; ten (10.5%) raw milk samples were found contaminated with tetracycline and sulfa drug residues.

Only 3 (3.1) out of 95 raw milk samples were found free of amphenicols, penicillin G, tetracycline and sulfa residues that could be detected using CT and CA tests used in this study.

Penicillin G is the mainly antibiotic found with a percentage of 62.1% (59) out of the 95 raw milk samples chosen arbitrary from the 220 double positive raw milk samples tested with MIT and were confirmed for inhibitors contamination.

Tetracycline is the second antibiotic found with a percentage of 43.2% (41) out of the 95 raw milk samples. Amphenicol is the third antibiotic found with a percentage of 40% (38) out of 95 raw milk samples, and sulfa drugs are the fourth antibiotics found with a percentage of 32.6% (31) out of 95 raw milk samples tested.

When ranking antibiotics by the mostly to the less used, penicillin G was the main antibiotic used in Lebanon dairy farmers based on the results of Charm® test with a percentage of 62.1%. Tetracycline is the second antibiotic used in the Lebanese dairy farms with a percentage of 43.2%. Amphenicols are the third antibiotic used in the Lebanese dairy farms with a percentage of 43.2% and Sulfa drugs are ranked fourth with a percentage of 32.6%.

6.7 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) TEST

6.7.1 Material and methods

High-performance liquid chromatography (HPLC) test was performed to validate results obtained by the microbiological test (MIT) and the immuno-chromatographic antibiotic residues rapid test (CT and CA test) that were performed in this study.

6.7.2 Chemicals used to perform HPLC test

- HPLC gradient grade acetonitrile and methanol were purchased from VWR chemicals.

- Oxalic acid and disodium hydrogen orthophosphate anhydrous were obtained from VWR chemicals.

- Citric acid anhydrous was purchased from HIMEDIA Laboratories.

- Disodium ethylenediamine tetra acetate (EDTA) obtained from MAY and BAKER LTD (Dagenham, England).

- Formic acid was purchased from Riedel de Haen, Sigma-Aldrich Laborchemikalien GmbH.

- Tetracycline HPLC graded; Lot: 180722-61 obtained from Pharmadex s.a.l.

- Florfenicol HPLC graded; batch number 201607067 obtained from Pharmadex s.a.l.

- Penicillin G potassium salt from Sigma-Aldrich.

- Ultra-pure water was used for the preparation of all aqueous solutions.

- Sodium Hydroxide (NaOH).

6.7.3 Instrumentation

All measurements were performed using an HP 1100 Series LC system (Hewlett Packard, Palo Alto, CA, USA) equipped with a quaternary pump, a vacuum degasser,

a column compartment, an auto sample and a diode-array detector. Hewlett-Packard ChemStation software was used by the instrument control and data processing utilities. The stainless analytical column was ODS hypersil C18, 5 μm (125 x 4mm) (from Hewlett Packard, Palo Alto, CA, USA).

For the preparation of the samples;

- a Vortex made by Daihan Scientific Co, Ltd (Korea) was used.

- a Spectrafuge 6C compact centrifuge (Edison, NJ USA) was used to separate the supernatant from the solid phase after protein precipitation.

- ultrasonic cleaner (BRANSON 200, made in Taiwan) was used to degas prepared mobile phase.

- pH meter CG 820 (SCHOTT GERATE, made in west Germany) was used to adjust pH of oxalic acid.

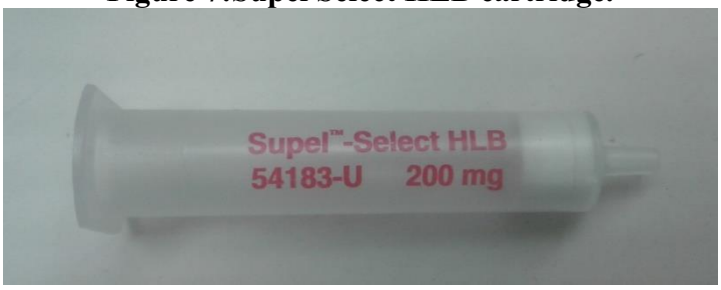
- Syringe membrane filters (0.45 μm) (Millipore, Ireland) and Speed SPE C18 solid phase extraction cartridges (Supel Select HLB) were used for processing samples.

6.7.4 **Pre-cleaning (Milk Samples Preparation)**

Ten ml of milk sample was taken in a 50 ml centrifuge tube. Add to it 10 ml of 0.1M EDTA-McIlvaine buffer (pH 4.0) followed by vigorous shaking for 5 mins. The sample was then centrifuged at 6000 rpm for 10 mins. The supernatant was collected and filtered through a Whatman filter paper 0.45 μm. Clean up of the extract was done by using Solid Phase Extraction (SPE) method. The filtrate was loaded on a Supel Select HLB cartridge preconditioned with 3 ml of methanol and 2 ml of water. The cartridge containing the sample was washed with 2 ml of water and then antibiotics

were eluted with 1.5 ml of methanol. The extract so obtained was filtered through a syringe filter (0.45 μm).

Figure 7:Supel Select HLB cartridge.



6.7.5 **Chromatographic conditions**

The LC gradient elution was performed using a mobile phase of water + 0.1% formic acid (eluent A), oxilac acid/ acetonitrile/ methanol (6:3:1) (eluent B), water/acetonitrile (75:25) + 0.1% formic acid (eluent C), and ACN/MeOH (2:1) + 0.1%formic acid (eluent D) at a flow rate of 1 mL/min. Note that oxalic acid concentration is 0.05 M and pH is 2.6 adjusted by NaOH (2M).

Chromatographic separation of the analytes was achieved with the following gradient shown in table 16. The autosampler and the column were maintained at 35 ºC. Quantitative measurements of the peak heights were performed by selecting the appropriate detection wavelength for the compounds to achieve maximum sensitivity. Therefore, florfenicol was quantified at 224 nm, penicillin at 210 nm, and tetracycline at 350 nm.

Table 16:Gradient program applied to florfenicol, penicillin and tetracycline

Time	Eluent A	Eluent B	Eluent C	Eluent D
0	40	0	50	10
2	40	0	50	10
3	0	0	90	10
6.5	0	0	90	10
7.5	0	90	0	10
8.5	10	50	30	10
10	40	0	50	10

Mobile phase was filtered and degassed by passage through a 0.45 μm nylon filter (Millipore) under a vacuum, and sonicated for 10 min. The flow rate was 1 ml/min, and the injection volume was 25 ml.

6.7.6 **Analytical method validation**

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice.

The validation parameters are: sensitivity, specificity, limit of detection, limit of quantification, linearity, accuracy, precision, repeatability, robustness and selectivity. The characteristics and the procedures used for validation were those described in the International Conference of Harmonization (ICH) Guidelines (ICH, 2015).

6.7.7 **Definitions of validation parameters technical word**

- **Sensitivity**

Sensitivity is the change in response on a measuring instrument divided by the corresponding change in stimulus.

- **Specificity**

The ICH documents define specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components (ICH, 2015).

- **Limit of detection**

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be detected but not necessarily quantitatively determined as an exact value. Based on the standard deviation of the response and the slope, detection limit may be expressed as follow (1).

(LOD) may be expressed as: LOD= 3.3(&/*s*) where,

&= the standard deviation of the response for the lowest concentration in the range

s = the slope of the calibration curve.

- **Limit of quantitation**

The quantification limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy. Based on the standard deviation of the response and the slope, the quantitation limit (LOQ) may be expressed as LOD=10(&/S) where:

&= the standard deviation of the response for the lowest concentration in the range

s = the slope of the calibration curve.

- **Linearity**

The linearity of an analytical procedure is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range.

- **Accuracy**

The accuracy of an analytical procedure is the closeness of test results obtained by that procedure to the true value. The accuracy of an analytical procedure should be established across its range.

- **Precision**

It is the degree of conformity between independent measurement results obtained under prescribed conditions.

- **Repeatability**

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

- **Selectivity**

The selectivity of a method of analysis refers to the degree to which the method of analysis is usable for determining the presence of specific analytical parameters in a complex mixture (matrix) without interference from other analytical parameters in the mix.

6.7.8 **Method Validation**

The HPLC-DAD method was validated for the determination of tetracycline, penicillin and florfenicol by assessment of following parameters: linearity, sensitivity, specificity, intra-assay and interassay precision, accuracy, LOD and LOQ.

The linearity and sensitivity of the proposed method were determined for all the test antibiotics from calibrations curves by plotting the peak height against increasing concentrations of each analyte under study ranging from 0.004 to 5μg/ml. Each concentration level was injected three times (n = 3). Linear regression data showed good linearity for all antibiotics with correlation coefficient (r^2) in the range of 0.997–0.999.

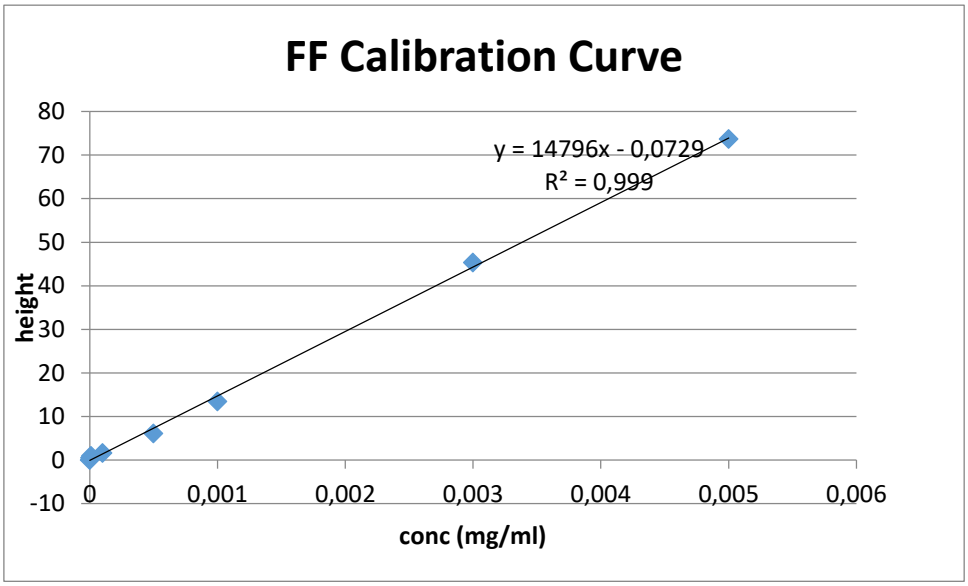


Figure 8: Florfenicol Calibration Curve

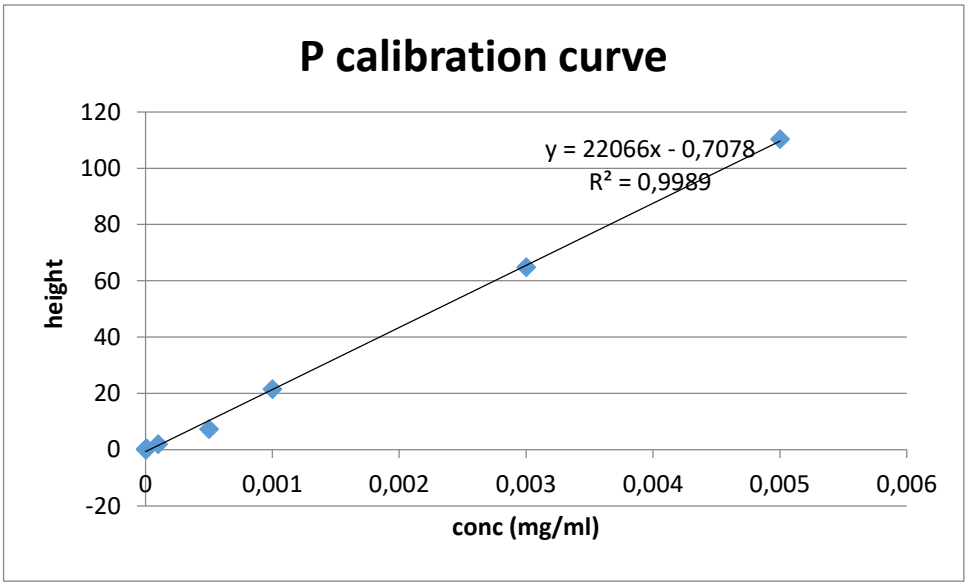


Figure 9: Penicillin calibration curve

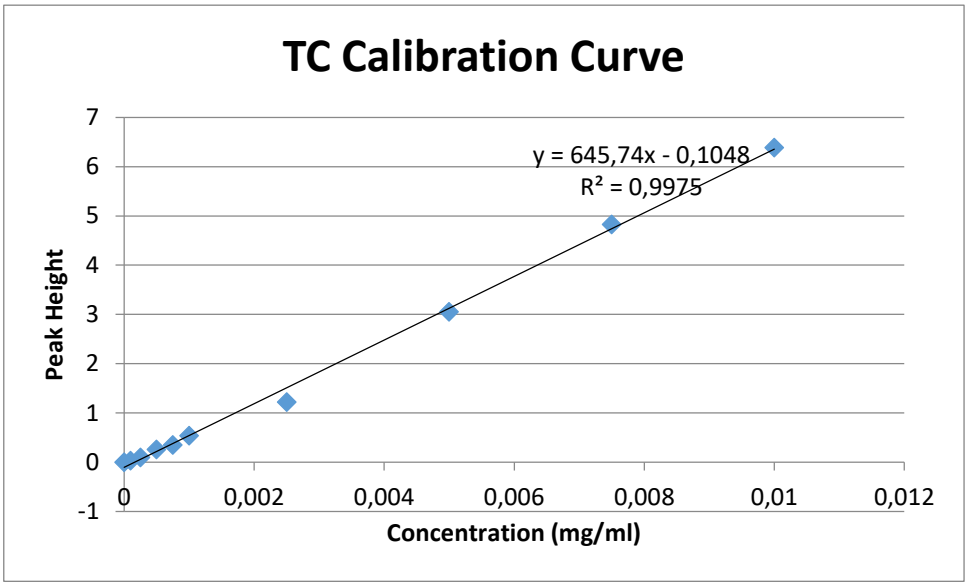


Figure 10: TC calibration curve

The application of the method to different blank milk samples in order to verify the method specificity demonstrated that no potential interferences from endogenous compounds were detected at 350 nm near to the retention time of tetracycline and at 224 nm near to the retention time of florfenicol.

The representative chromatograms of blank milk samples and samples spiked with 0.001 mg/mL of antibiotic standards are shown in Figure 11 and 12.

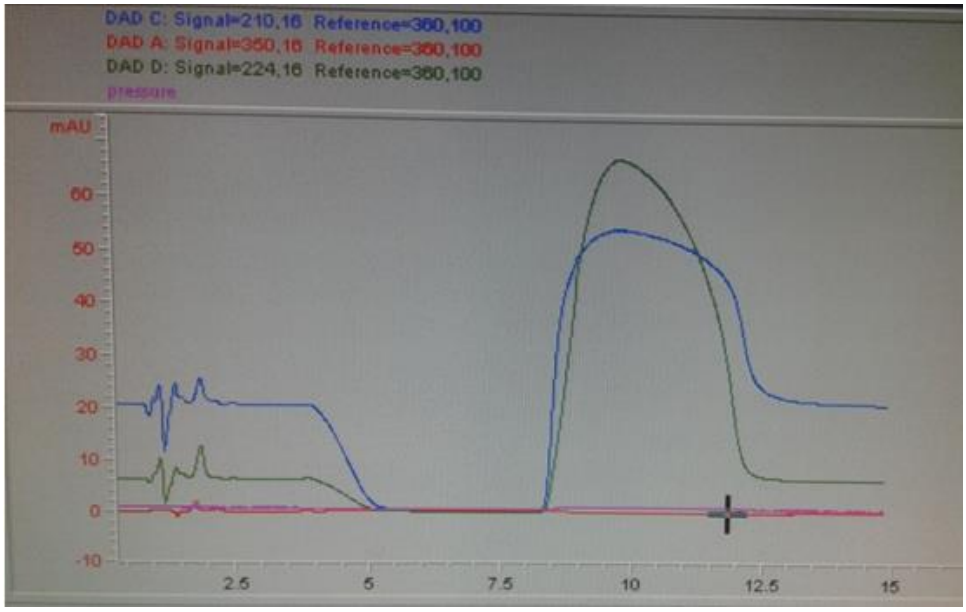


Figure 11: Blank milk sample

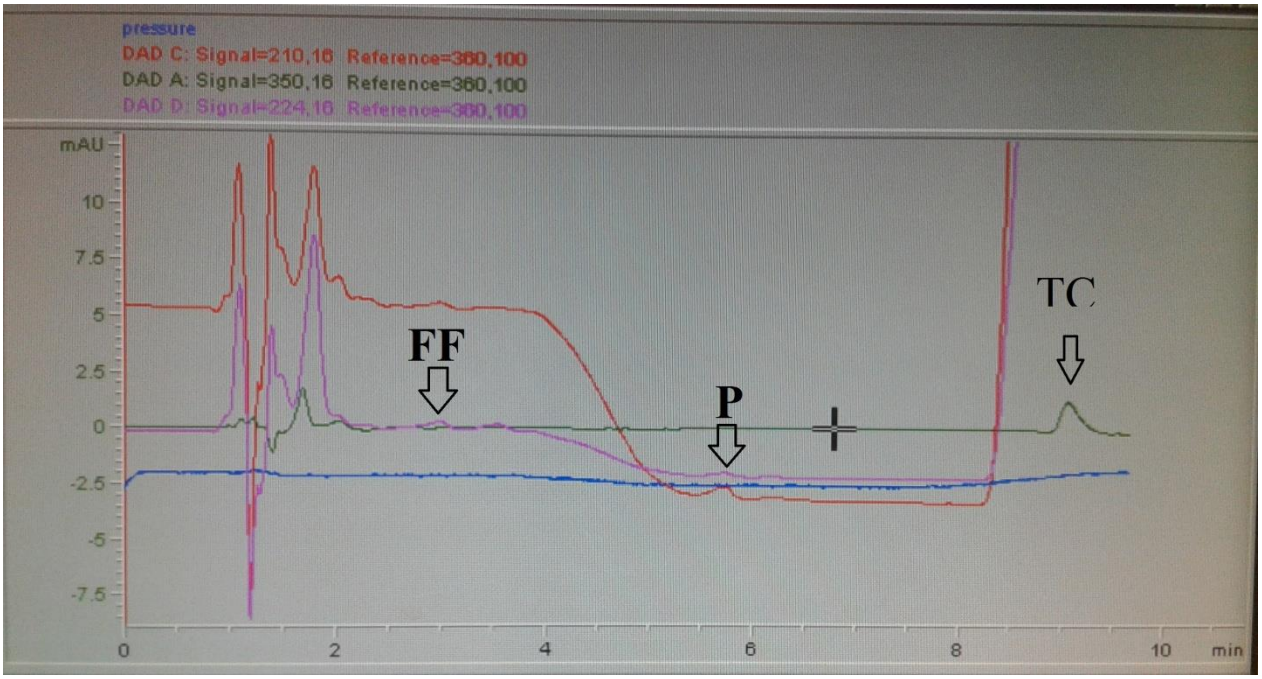


Figure 12: Fortified milk sample with florfenicol, penicillin and tetracycline (FF: florfenicol; P: Penicillin; TC: tetracycline)

Since no characteristic interferences were detected at retention time of each analyte, the optimized method presents adequate selectivity for the determination of targeted antibiotics.

The LOD and the LOQ were defined as the concentrations obtained calculating the standard deviation of the lowest range multiplied by 3.3 and ten times respectively. The LOD and LOQ values are summarized in the table 17 below.

Table 17:LOD and LOQ results

Antibiotic	LOD (mcg/l)	LOQ (mcg/l)
Florfenicol	12.4	38
Penicillin	6	18
Tetracycline	15	45

The precision of the method was evaluated as intra-assay and inter-assay and expressed as % of relative standard deviation (RSD) of peak height measurements. Repeatability of the method was tested by six replicate injections of spiked milk. Intra- and inter-day variations of retention times and concentrations, expressed in RSD %, are listed in Tables 18 and 19.
Very low variation was observed in the retention times, with RSD values not exceeding 1.65%. RSD values for antibiotic concentration ranged from 0.77 and 1.68%. According to the European Commission Decision 2002/657/EC, the intra-assay precision and inter-assay precision should be lower than 15% and 23%, respectively, and the observed values are in agreement with the EU guidelines.

Table 18:Intraday precision of retention time and concentration of different antibiotics in spiked milk

Antibiotic	**Spiked milk (c= 0.005 mg/ml)**			
	Retention time		Concentration	
	min	%RSD	mg/ml	%RSD
Florfenicol	2.82	0.28	0.00501	0.15
Penicillin	5.62	0.18	0.00502	0.7
Tetracycline	9.088	0.033	0.00475	0.83

Table 19:Interday precision of retention time of different antibiotics in spiked milk

Antibiotic	**Spiked milk**				
	Retention time (min)				
	Day 1	Day 2	Day 3	Mean(min)	%RSD
Florfenicol	3.04	2.78	2.82	2.88	0.36
Penicillin	5.85	5.62	5.58	5.69	1.65
Tetracycline	9.107	9.088	9.093	9.096	0.046

Table 20 :Interday precision of concentration of different antibiotics in spiked milk

Antibiotic	Spiked milk				
	Concentration (0.005mg/ml)				
	Day 1	Day 2	Day 3	Mean(mg/ml)	%RSD
Florfenicol	0.00501	0.005014	0.004988	0.005005	0.77
Penicillin	0.00513	0.00506	0.00507	0.00508	1.66
Tetracycline	0.00473	0.00439	0.00625	0.00514	0.815

The accuracy of the method was determined by triplicate analysis of spiked milk samples at three fortification levels. The recovery for studied antibiotics ranged from 82 to 111.54 %. The results are presented in Table 20 and 21. The recovery values are in accordance with the EU guidelines, which established a range of 80–120% for these concentration levels (European Commission, 2002).

Table 21:Accuracy of method 2

Accuracy (% Recovery)			
Level	**Florfenicol**	**Penicillin**	**Tetracycline**
Low	93.29	86	82
Medium	95.14	107	109
High	111.54	83	85

Table 22: maximum residue limits (MRLs) and risk management recommendations (RMRs) for residues of veterinary drugs in foods (Codex Alimentarius, 2018)

Antibiotic	Species	Tissue (µg/l)	MRL (µg/kg)	CAC	Acceptable Daily Intake
Penicillin G	Cattle	Milk	**4**	23 (1999)	30 µg-penicillin/person/day (JECFA50). Residues of benzylpenicillin and procaine benzylpenicillin should be kept below this level.

Sulfadimidine	Cattle	Milk	**25**	21 (1995)	0-50 µg/kg bw (JECFA42)
Tetracycline	Cattle	Milk	**100**	26 (2003)	Group ADI for chlortetracycline, oxytetracycline and tetracycline: 0-30 µg/kg bw (JECFA50). Group ADI for chlortetracycline, oxytetracycline and tetracycline
Phenicols	Cattle	Milk	"**no safe level** of residues of chloramphenicol or its metabolites in food that represents an acceptable risk to consumers"	37 (2014)	Recommended risk management measures in view of the JECFA conclusions on the available scientific information, there is for this reason, competent authorities should prevent residues of chloramphenicol in food. This can be accomplished by not using chloramphenicol in food producing animals.

6.7.9 Results of HPLC-DAD

Table 23:Showing results of the tested samples using HPLC-DAD

Antibiotics		Milk (µg/kg)	Number of positive samples	Percentage of contaminated samples
Penicillin G, tetracycline and florfenicol		Between 5 - 1565	**143** out of 220	**65**
Separated results	**Penicillin G**	Between 5 – 311	**118** out of 220	**53.6**
	Tetracycline	Between 166 -1565	**51** out of 220	**23.1**
	Florfenicol	Between 5 - 190	**50** out of 220	**22.7**
Penicillin, teteracycline and florfenicol		Between 8 - 242	**6** out of 220	**2.7**
Penicillin and tetracycline		Between 5 - 1565	**25** out of 220	**11.3**
Penicillin and florfenicol		Between 5 - 311	**25** out of 220	**11.3**
Florfenicol and tetracycline		Between 6 - 169	**2** out of 220	**0.9**

6.7.10 **Interpretation**

Out of 220 tested dairy raw milk samples, 143 (65%) samples were found contaminated with penicillin G, tetracycline and florfenicol with a concentration above the European maximum residue limit that range between 5 and 1565 µg/kg. One hundred and eighteen (53.6%) dairy raw milk samples out of 220 double checked samples with Delvotest, were found to be contaminant with penicillin G residues with a concentration above the European maximum residue limit (4 µg/kg) that range between 5 µg/kg and 311 µg/kg. Fifty-one (23.1%) dairy raw milk samples out of the 220 samples were found to be contaminant with tetracycline residues with a concentration above the European maximum residue limit (100 µg/kg) that range between 166 µg/kg and 1565 µg/kg. Fifty (22.7%) dairy raw milk samples out of the 220 samples were found to be contaminated with florfenicol residues with a concentration above the minimum required performance limits (MRPL) (0,3 µg/kg) that range between 5 µg/kg and 190 µg/kg.

Twenty-five of the 220 tested samples were contaminated at the same time with penicillin G and tetracycline residues with a concentration above the European maximum residue limit.

Twenty-five of the 220 tested samples were contaminated at the same time with penicillin G and florfenicol residues with a concentration above the European maximum residue limit and minimum required performance limits.

Six of the 220 tested samples were contaminated at the same time with penicillin G, tetracycline and florfenicol with a concentration above the European maximum residue limit and minimum required performance limits.

Two of the 220 tested samples were contaminated at the same time with tetracycline and florfenicol residues with a concentration above the European maximum residue limit and minimum required performance limits.

The antibiotic residue that is mostly found in the 220 tested samples is penicillin G with 118 non-compliant samples. Those findings are followed by tetracycline residues with 51 non-compliant samples and at the end with 50 non-compliant samples for florfenicol.

The minimum concentration found above the maximum residue limit and minimum required performance limits was 5 µg/kg for penicillin G and florfenicol residues. The maximum concentration found above the maximum residue limit was 1565 µg/kg for tetracycline residues.

6.8 GENERAL DISCUSSION ABOUT ANTIBIOTIC RESIDUES IN COW'S MILK IN LEBANON

The questionnaire has proved that with their knowledge level, the Lebanese farmers are not able to use veterinary antibiotics prudently. Moreover, without considering the high amount of drug residues in any unrecovered animal, the latter is sold for meat production, proving that farmers are not aware of the antibiotic residues effect on consumer's health, and environment.

In this study, only 40% know that antibiotic is used for bacterial infections and 53% believe that it is used for viral and bacterial infections as well and 7% believes that antibiotics cure only viral infections. Results are higher than the one found in Yasin et al, (2019) study to determine knowledge, attitudes, and behaviour of farmers dealing with animal husbandry in eastern Turkey, where 11% of the asked farmers assumed that antibiotics don't kill bacteria.

Moreover, only 31% of the questioned farmers in Lebanon believe that frequent use of antibiotics will decrease the efficacy of treatment the more they are used. Those results are lower than what Friedman *et al.* (2007) has found in his study about dairy farmers at the South of Carolina where 70% of questioned farmers agreed to that. The Lebanese farmer's answers proves that the knowledge level about antibiotics is very low, those results are similar to results found in a study done by Tola *et al.* (2017) where 76.9 % dairy farms had no knowledge about the drug they have been using and did not use prescribed drugs.

In our survey, 58% of the questioned believe that antibiotics will speed up the recovery of any illness in cows, but in the cross-sectional study that was done by Tola et al. (2017) to determine the prevalence and assess the level of awareness of dairy farm owners at Bishoftu town dairy farms using a scale of 5 points merging from totally agreed to totally disagreed, 9% totally agreed and 31% agreed that antibiotics can be used for all types of diseases.

Results show that farmer's knowledge concerning antibiotic usage is limited and primitive.

The main antibiotics used in the selected farms were penicillin (37%), florfenicol 34%, tetracycline (25%) and quinolone (4%). The results are not similar to those found in a study done by Sawant *et al.* (2005) from July 2001 to June 2002 on antibiotic usage of 113 dairy herds from 13 counties in Pennsylvania, where Beta-lactams was mostly used followed by tetracyclines.

Ninety percent of the selected farmers get their information about antibiotics usage from professional recommendations but 10% receive it from an unprofessional recommendation. Those results are higher than results found in a study done by Tola *et al.* (2017) where 76.9 % dairy farms had no knowledge about the drug that they have

been using and did not use veterinarian prescribed drugs. Moreover, results are extremely greater than Tesfaye (2007) study results in Nazareth, East Shoa, where 3.9% of farmers were using antibiotic without prescriptions. Furthermore, Yasin *et al*. (2019), has reported in his study at eastern Turkey, by using a scale of 5 points merging from totally agreed to totally disagreed, has found that 11% totally agreed that they take recommendations from other farmers and 53% of the questioned farmers agreed as well.

Sixty-eight percent of the farmers have never checked antibiotic leaflet instruction and 32% check it occasionally. Results are higher than what Yasin *et al*. (2019) has found in his study at eastern Turkey were 11% of the farmers totally agreed that they do not check the leaflet and 26% agreed.

It is clear in our study that 90% of the farmers rely on their source of antibiotics to get information concerning antibiotic use. Results are higher than found by Yasin *et al*, (2019) at eastern Turkey, where 11% of the farmer totally agreed on accepting recommendations from other farmers and 53% agreed. However, 63% of the farmers stopped the treatment directly after symptoms disappeared, 12% after a few days regardless of the outcome and 4% after antibiotics ran out. Our study results are higher that Yasin *et al*. (2019) has found in his study at eastern Turkey were 14% of the farmers totally agreed on stopping antibiotics directly after the first day of symptoms disappears and 45% agreed. Not only but in our study, 3% of the farmers changed antibiotic treatment when the prescribed drug ran out, knowing that 95% have used the same antibiotic molecule without knowing, during the same treatment course. Results are different than what Sawant *et al*. (2005) has found in dairy herds in Pennsylvania where 24% of questioned producers complete the course of antibiotic treatment.

All of the questioned farmers have used antibiotics before consulting the veterinarian, but in Yasin *et al*. (2019) study at eastern Turkey, 12% totally agreed and 36% agreed to start treatment before consulting the veterinary doctor. Results are higher than what Tesfaye (2007) has reported in his study at Nazareth East Shoa where only 3.9% of farmers were using antibiotic without prescriptions.

Forty-five percent of the Lebanese dairy farmers change antibiotic dosage deliberately during a treatment and 55% do it sometimes. Results are totally different from what Sawant *et al*. (2005) has found in dairy herds in Pennsylvania where only extra-labelled use of antibiotics are administered when consulted by a veterinarian on the majority of the questioned farms.

Furthermore, 68% of the Lebanese farmers have never checked the antibiotic leaflet and 32% did it sometimes. Compared to Turkish farmer's in Yasin *et al*. (2019) study 11% totally agreed and 26% agreed that they do not check the drug leaflet.

When the farmers were asked: when do they stop antibiotic treatment course, 63% answered that when symptoms disappeared, 14% after a professional recommendation, 12% after few days of the treatment, 7% after the recovery of the animal and 4% when the antibiotic ran out. Moreover, all farmers approved that antibiotics can cure bacterial infections. Their access to antibiotics is very easy. Furthermore, all of them use antibiotics without veterinary prescription, which is different from what Yasin *et al*. (2019) has found in his study where 12% of the questioned farmers totally agreed with it and 36% agreed. Those results defer from what Friedman *et al.* (2007) has found where 50% of the dairy farmers in South Carolina complete the course of the treatment as prescribed by the veterinarian.

Results if this research are basically high, but when translating those results on a level that can be touched physically, the way of imaging the situation will be clearer.

For instance, the quantity of milk produced in the 7 Lebanese Governorates from the biggest and medium dairy farms with the quantity of milk collected by the center of collection, was given by the Ministry of Agriculture statistics in 2018. Unfortunately, there is no online data about those numbers.

- In the Beqaa Valley, 412,092 litre of dairy raw milk are produced daily;

- in Baalbek-Hermel, 297,360 litre of dairy milk are produced daily;

- in Akkar, 242388 litre of dairy raw milk are produced daily;

- in Mount Lebanon, 171,000 liters of dairy raw milk are produced daily;

- in Nabatiyeh, 122,832 litre of dairy raw milk are produced daily;

- in the North, 108,540 litre of dairy raw milk are produced daily and in the South, 84168 litre of dairy raw milk are produced daily.

This production is multiplied by 30 days to obtain the total quantity of raw daily milk produced in each Governorates per month.

- In the Beqaa Valley, 12,362,760 litre of dairy raw milk are produced monthly;

- in Baalbek-Hermel, 8,920,800 litre of dairy milk are produced monthly;

- in Akkar, 7,271,640 litre of dairy raw milk are produced monthly;

- in Mount Lebanon, 5,130,000 liters of dairy raw milk are produced monthly;

- in Nabatiyeh, 3,684,960 litre of dairy raw milk are produced monthly;

- in the North, 3,256,200 litre of dairy raw milk are produced monthly and in the South, 2,525,040 litre of dairy raw milk are produced monthly.

Table 24:showing the quantity of dairy milk produced and contaminated with inhibitors

Month/Governorate	Mount Lebanon n=24	Beqaa Valley n=42	Baalbek/ Hermel n=11	North n=5	South n=5	Nabatiyeh n=8	Akkar n=7
Total numbers of positive samples per Governorate	39	99	37	12	4	16	13
Total number of collected samples	1020						
Total number of positive samples using MIT	220						
% of positive samples per governorate	16.575	24.043	34.309	24.48	8.16	20.4	18.94
Quantity of milk (L) per day	171 000	412 092	297 360	108 540	84 168	122 832	242 388
Quantity of milk (L) per Month	5 130 000	12 362 760	8 920 800	3 256 200	2 525 040	3 684 960	7 271 640
Quantity of contaminated milk (L) per Month	3 095.022624	5 141.967914	2 600.127186	1 330.14706	3 094.4118	1 806.352941	3 838.72398
Total Quantity (L) of contaminated milk per month	**20 906.75347**						

Based on the results of MIT, and the statistics of the Ministry of Agriculture, the quantity of milk contaminated with inhibitors in the Beqaa Valley is 12,362.76 tones. In Baalbek-Hermel, 8,920.8 tones, in Akkar, 7,271.64 tones, in Mount Lebanon, 5,130 tones, in the North, 3,256.2 tones and in the South 2,525.04 tones.

The total quantity of dairy raw milk contaminated with inhibitors above the European maximum residue limit during one month in the 7 Lebanese Governorates is 20.9 tons. In one year, 250.8 tons of dairy raw milk is contaminated with inhibitors

with a concentration above the European maximum residue limits. Our results show that 220 (22 %) of dairy raw milk produced in Lebanon is contaminated with inhibitors with a concentration above the European maximum residue limits.

The lowest percentage of dairy milk contamination found in Lebanon is in the South. Those results might be due to the rules applied by the biggest dairy plant (Milco) in this Governorate that buys milk from all the biggest farmers in this Governorates. This dairy factory checks milk for beta-lactam and tetracycline residues using a rapid test. Not only, but in their rules, Hassoun & Chamaa co. (Milco) have established a contract with all the farmers from whom they buy raw milk. This contract obliges the farmer to pay 10,000 Lebanese Liras (6,666 USD) in case of antibiotic residues were found in his milk. Apparently, the protocol have obliged the farmer to manage the quality of his milk before selling it to the dairy plant.

In Constantine region (North East Algeria), a study established by Boultif *et al*. (2016) using MIT to identify inhibitors contaminating commercialized milk. Result shows that 25% (30 from 120) of tested cow milk were contaminated with inhibitors and 15% (18 out of 120) were doubtful.
In another study that was established by Aggad *et al*. (2009) using MIT in the west of Algeria, it had been shown that 24 (28.9%) out of 83 cow milk samples were found positive for antibiotic residues.

Another study established in Tiaret, Algeria by Guetarni *et al*. (2006) using MIT shows that 26.38 % positive samples were contaminated with antibiotic residues. Moreover, Srairi *et al*. (2006) has found in Morocco 26% of contaminated milk samples out of a total of 109 bulk milk samples obtained directly after milking from 109 different farms around the city of Rabat-Salé and tested with MIT.

In Montenegrian dairies, Nikolić *et al*. (2011) found that 478 samples (7.84 %) of 6161 samples of raw milk collected during a period of six months were tested using MIT and was found positive for inhibitors.

Ardiç and Durmaz (2006) has found positive 32% (96 samples) out of 300 milk samples tested for inhibitors in Sanliurfa, Turkey, using Bacillus stearothermophilus as the sensitive microorganism.

In Ethiopia, between October 2007 and May 2008, a study was performed by Desalegne Abebew Syit where 34 (8.5%) out of 400 milk samples were found positive for antibiotic residue using Delvotest®. Moreover, Díez *et al*. (2013) has found in Colombia that 12.8% (40) out of 272 milk samples were found positive for inhibitors using MIT.

MIT analysis showed that the percentage of inhibitors in dairy raw milk in Lebanon (22%) is not the highest when comparing it to other studies, but it poses a high risk for human health and dairy industries.

Concerning CT and CA results, 92 (96.8%) samples out of 95, were found contaminated with antibiotic residues with a percentage above the European maximum residue limit.
When comparing the CT and CA results with the same samples tested using HPLC, only 6 (6.3%) samples showed different results out of 95 samples, but the 6 samples are contaminated with at least one of the antibiotic residues in search. Moreover, 38 (40%) dairy raw milk samples were found contaminated with amphenicols, 59 (62.1%) raw milk samples were found contaminated with penicillin G, 41 (43.2%) raw milk samples were found contaminated with tetracycline and 31 (32.6%) raw milk samples were found contaminated with sulfa drugs.

Few studies are performed on dairy raw milk to detect antibiotic residues using CT and CA tests.

Comparing our results with a study that was done by Alomirah *et al*., (2007) in Kuwait were samples are collected from 30 local dairy farms from April 2004 to February 2005 and tested using Charm test. Results shows that 62 samples (20.1%) out of 308 samples were positive for beta-lactam. One hundred twenty-one (37.3%) out of 324 samples were positive for tetracycline and 61 (29.4%) out of 207 samples were positive for chloramphenicol. All positive samples described were above the maximum residue limits allowed. In total, 29.1% of the tested local raw milk samples exceed the European maximum residue limits for the tested drugs with a predominance of tetracycline (Alomirah *et al*., 2007).

Another study was performed at Sixteen cities in the United States and four in Canada, were 174 dairy milk samples were collected from the states and 40 from Canada. Using Charm test, results show that 150 out of 174 samples were positive for antibiotics. Eighty-two samples were contaminated with sulphamethazine and 48 with tetracycline. For the Canadian samples, 12 samples were contaminated with tetracycline and another 12 with sulphamethazine (Collins-Thompson *et al*., 1988).

High-performance liquid chromatography (HPLC) test that is known as one of the most sensitive tests have shown that out of 220 tested dairy raw milk samples, 143 (65%) samples were found contaminated with penicillin G, tetracycline and florfenicol with a concentration above the European maximum residue limit that range between 5 and 1,565 μg/kg.

Penicillin G was found in 53.6% of the tested samples, tetracycline in 23.1% and florfenicol in 22.7% of tested samples. A combination of penicillin G, tetracycline and florfenicol was found in different samples in a percentage of 26.2%. (58 out of 220).

All antibiotic residues found was above the European maximum residue limit with a concentration that range between 5 and 1565 μg/kg.

On field level, the risk of dairy milk contamination is on a high level. For instance, the 7 Lebanese Governorates produce an average of 43,151.4 tons of dairy raw milk each month. The contamination level of dairy raw milk in Lebanon by antibiotic residues (penicillin G, tetracycline and florfenicol) is 65%. Results reveal that 28,048.41 tons of dairy raw milk is contaminated by penicillin G, tetracycline and florfenicol residues in Lebanon, with a concentration above the European maximum residue limit.

Comparing our research with another study that was performed using HPLC between October 2007 and May 2008 to detect and determine oxytetracycline and penicillin G residue levels in bulk milk of cows in Debre Zeit dairy farms. Results show that 34 (8.5%) out of 400 raw milk samples were found positive for tetracycline and 8 for penicillin G residues with a concentration above the maximum residue limit (Abebew *et al*., 2014).

A study was done in Ankara-Turkey to detect antibiotic residues in raw and pasteurized milk products using TLC (Thin Layer Chromatography)/Bioautographic method. Out of 240 milk samples 5 (1.25%) samples were non-compliant with a concentration above the European maximum residue limits (Ergin Kaya and Filazi, 2010).

A study was performed in India were a total 100 milk samples were collected vendors, from Hisar and nearby areas. Using HPLC UV-VIS, results show that chlortetracycline residues were found in 9 samples, oxytetracycline residues in 6 samples and tetracycline in 3 samples were 5 samples were non-compliant with oxytetracycline residues above the European maximum residue limit (Chauhan *et al*., 2019).

Moreover, in peri-urban Nairobi, Kenya a study was performed to determine the prevalence of antibiotic residues in raw milk from dairy farms using HPLC. Results show that out of 139 milk samples collected from 87 farms, 19.6 % (27/139) were contaminated with antibiotic residues with a majority that exceed the maximum residue level (Wambua, C.N. 2016).

Another study was done in small scale dairy farms in Bagamoyo district, Tanzania. Collected samples were tested by using HPLC Technique. Results show that the prevalence of oxytetracycline residues was 10% (11) out of 110 raw dairy milk samples with a mean value level around 766.3± µg/l (Ramadhani, 2015).

6.9 CONCLUSION

This research was aiming to understand the knowledge level of the Lebanese dairy farmers, find out the prevalence and identify antibiotic residues in dairy cow's bulk tank milk produced in Lebanon. Concerning the prudent use of veterinary antibiotics, the results of the study's questionnaire have shown that most of the Lebanese dairy farmers have a low knowledge level. This was also reflected in the results of the 1020 tested dairy raw milk samples that were collected monthly from March 2018 until December 2018 from the 7 Lebanese Governorates that endorse mainly medium and big dairy farms in Lebanon. Collection was proportional to the dairy farms distribution on the Lebanese lands. After performing three tests, 220 samples were found double positive using a microbiological test (Delvotest®□)with inhibitors that exceed the European maximum residue limits. The 220 samples were tested using high-performance liquid chromatography test (HPLC-DAD). The aim of

the test was to detect the main three antibiotics (penicillin G, tetracycline and florfenicol) that were mainly used by the questioned farmers.

Sixty-five percent (143) positive results, out of the 220 samples, were found with a concentration above the European maximum residue level. Out of those 143 positive samples 53% were contaminated with penicillin G with a concentration that ranges between 5 and 311 µg/kg that is above the European maximum residue level. 23% of them were also found contaminated with tetracycline with a concentration that ranges between 166 and 1565 µg/kg, and 22.7% were contaminated with florfenicol with a concentration that ranges between 5 and 190 µg/kg.

Ninety-five positive samples tested with HPLC-DAD were tested using immuno-chromatographic antibiotic residue rapid tests Charm® TRIO and Charm® AMPH tests. Results showed 96.8% positive results where sulfa was found marking a percentage of 32.6%, penicillin G 62.1%, tetracycline 43.2% and amphenicol 40%. All samples were contaminated with a concentration above the European maximum residue limits.

The found results revealed a high level of veterinary antibiotic residue contamination in dairy raw milk produced in the Lebanese dairy farms that covers most of the Lebanese market. As discussed previously in the thesis concerning the Lebanese daily food basket that rely on dairy products, results highlighted a potential public health problem. Furthermore, the results have proved that the dairy farmers require training activities concerning the prudent use of antibiotics. Moreover, a national pilot plan aiming to control veterinary drug residues is necessary. This plan should cover dairy raw milk from farmer to consumer, similar to the actual working system that I have experienced during my visits to Sardinia. The plan should aim to control veterinary drugs at selling points to veterinary prescriptions, dairy plants checks and

verification activities. The pilot plan should be monitored by competent and qualified people that are aiming to increase the Lebanese dairy milk quality and help in protecting the consumers from antibiotic residues in food, increasing the dairy sector quality and minimising the imprudent use of veterinary antibiotics.

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Annex

Table 25: subjects and questions of the questionnaire

Subjects of the questionnaire	Questions
1. General information	1. Are you the treatment handler? 2. What do you do with the animal if the treatment failed? 3. What do you do with milk during antibiotic treatment? 4. What does the milk with antibiotics used for? 5. When using antibiotics that is excreted in milk production do you usually throw the milk? 6. What do you care more about when using antibiotic that is excreted in milk, selling the milk or human health? 7. How much do you sell 1L of milk containing antibiotics? 8. Do you know the quantity of AB used in your farm per year? If yes, how many?
2. Farmer knowledge about antibiotics	1. Do you know what an antibiotic is? 2. What are antibiotics used for? 3. Can antibiotics cure bacterial infections? 4. Can antibiotics cure viral infections? 5. Do you think the use of antibiotics will speed up the recovery of any illness in cows? 6. Do you think frequent use of antibiotics will decrease efficacy of treatment when using the antibiotic again? 7. Have you heard of antibiotics resistance? 8. Is the efficacy better if the antibiotics are newer and cost more? 9. Do you know about agonist and antagonist antibiotics?

	10. Have you ever seen any adverse reaction when you were using antibiotics? 11. What is (are) the common adverse reaction(s) of antibiotics? 12. What do you do for the adverse reactions? 13. Do you think you can treat common infectious diseases successfully by yourself?
3. Main three antibiotics used at farms	1. What are the main three antibiotics that you use in your farm?
4. Access to antibiotics	1. How difficult is your access to antibiotics? 2. From where do you buy antibiotics that are required by a veterinary prescription? 3. From where do you buy antibiotics for self-treatment?
5. Information about antibiotic usage	1. What is your information source of antibiotics usage? 2. How do you find your access to your source of information?
6. Method of antibiotic usage	1. Have you ever used antibiotics without consulting a veterinarian? 2. What is the reason of using antibiotics without consulting a veterinarian? 3. What was your selection of antibiotic based on? 4. What did you consider when selecting antibiotics? 5. Did you ever check the instruction leaflet inside the antibiotic package in case of self-treatment? 6. How much do you understand the instructions on the leaflet? 7. How do you know the dosage of antibiotics?

	8. Did you ever change the dosage of antibiotics deliberately during the course of self-treatment? 9. Why did you change the dosage of antibiotics during the course of self-treatment? 10. Did you ever switch antibiotics during the course of self-treatment? 11. Why did you switch antibiotics during the course of self-treatment? 12. How many different antibiotics did you use maximally during a single illness? 13. Have you ever found that you used the same antibiotics with different commercial names during the same treatment course? 14. Do you usually choose an antibiotic that is not excreted in milk production? 15. When do you normally stop using antibiotics during treatment?