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The effect veterinary antibiotic residues in milk have on yoghurt making

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1. Abbreviations

The following abbreviations were used in this thesis.

CM	clinical mastitis
CNS	coagulase-negative staphylococcus
ESI(+)	(positive) electrospray ionisation
FDA	Food and Drug Administration of the USA
HIV	human immunodeficiency virus
LC-MS/MS	liquid chromatography coupled with tandem mass spectrometry
LOD	lowest level of detection
LOQ	lowest level of quantitation
LPS	lipopolysaccharide
MRM	multiple reaction monitoring
RNA	ribonucleic acid
SCC	somatic cell count
TAR	trans-activating region
TD	test day
tRNA	transfer ribonucleic acid

2. Introduction

The dairy industry is very important industry in Ireland. It is estimated that the dairy industry is worth €2.23 bn in exports alone (DAFM, 2016), Ireland produces enough dairy produce to feed 52 million people. Farming is a vital part of the Irish economy with 116,400 people employed in the industry (CSO, 2016).

The domestic market is also very valuable, the domestic yoghurt industry is worth €310 million euro (Bord Bia, 2017). This study is aimed at showing the importance of following antibiotic withdrawals by showing the effects the presence of antibiotics can have on the yoghurt making process.

An important disease in the dairy industry in mastitis. Mastitis can be described as clinical or sub clinical. Clinical mastitis is displayed by clinical signs and can be systemic and well as being localised to the udder. Subclinical mastitis does not show sign to the naked eye but can be seen in a raised somatic cell count (SCC).

For my study neomycin (aminoglycoside) and tetracycline (broad spectrum polyketide) were chosen. These are the active ingredient of the veterinary formulation called Mastijet, an intramammary infusion targeted for lactating cows. The tests were conducted by adding these antibiotics to the milk in concentrations that has been measured in treated cow's milk. Three different antibiotics levels were tested, low, medium and high in an attempt to replicate the levels of contamination in the milk in the 3 days after administration of the medication. The neomycin and tetracycline levels have been determined in the milk itself, before and after pasteurization, as well as, in the yoghurt prepared from it.

I carried out the yoghurt making process. Yoghurt is a fermented milk product that contains the characteristic bacterial cultures Lactobacillus bulgaricus and Streptococcus thermophiles. The milk was held at 42°C until a pH 4.5 is reached.

The aim of the study is to see if a residue of antibiotic in the milk will affect the culturing process of yoghurt. I recorded the antibiotic levels in the yoghurt, the pH and the physical properties of the yoghurt. This was in an attempt to see if the yoghurt cultured as normal or there were some sort of abnormalities.

3. Literary review

3.1. Mastitis

Mastitis is the inflammation of the mammary gland and udder tissue, and is a major endemic disease of dairy cattle. It usually occurs as an immune response to bacterial invasion of the teat canal by variety of bacterial sources present on the farm, and can also occur as a result of chemical, mechanical, or thermal injury to the cow's udder. Milk-secreting tissues and various ducts throughout the udder can be damaged by bacterial toxins, and sometimes permanent damage to the udder occurs. Severe acute cases can be fatal, but even in cows that recover there may be consequences for the rest of the lactation and subsequent lactations (AHDB, 2017).

Mastitis is an extremely costly disease to dairy farmers, due to cost of treatment and loss of production and fatalities. They are also more likely to need to be culled earlier and may lose body condition. In 2013 mastitis was estimated to cause a net farm profit decrease of €19,504 for a 40 hectare farm (ICBF, 2017).

The cost of clinical mastitis (CM) was studies in the United States. It found the average cost of CM per cow and year in the herds in the study was $71. The average cost of a CM case was $179. It was composed of $115 because of milk yield losses, $14 because of increased mortality, and $50 because of treatment-associated costs. The estimated cost of CM was highly dependent on cow traits: it was highest ($403) in cows with high expected future net returns (e.g., young, high-milk yielding cows), and was lowest ($3) in cows that were recommended to be culled for reasons other than mastitis. The cost per case of CM was 18% higher with a 20% increase in milk price and 17% lower with a 20% decrease in milk price. The cost per case of CM was affected little by a 20% change in replacement cost or pregnancy rate. (Bar, et al., 2008)

Maintenance of a clean and dry housing environment and implementation of proper milking procedures are essential for prevention of mastitis. All quarters should be fore stripped, which will begin the milk let-down process. Following fore stripping, the use of an FDA-approved and efficacious pre-milking teat disinfectant is particularly important for this mastitis-causing pathogen. The pre-milking teat disinfectant should remain on the teats for 30 seconds prior to removal with either a paper towel or a single-use clean and dry cloth towel. Following these guidelines, the time from the start of manual stimulation (fore

strip or wipe) to unit attachment should be in the range of 60 to120 seconds. This will allow the appropriate time for milk let down.

The use of blanket dry cow therapy with a long-lasting antibiotic preparation is an effective way to cure existing infections at drying off. Furthermore, studies report the use of an internal teat sealant will significantly reduce the new intramammary infection (acquired during the dry period) caused by environmental streptococci, which includes S. uberis. The use of an internal teat sealant in conjunction with blanket dry cow therapy will double the cost of dry cow treatment. However, in herds where environmental streptococci are the predominant bacteria isolated from clinical mastitis, this is an effective way to prevent new infections and cure existing infections during the dry period (Petersson-Wolfe & Currin, 2012).

3.1.1. Clinical Mastitis

Mastitis is generally classified as clinical or subclinical depending on the degree of inflammation in the mammary gland. Clinical mastitis is characterized by visible abnormalities in the milk or the udder. The most obvious abnormalities in the milk are flakes, clots and a watery appearance. Abnormalities associated with the udder are heat, swelling and sensitivity to touch. Cows with severe clinical mastitis typically have sudden onset of udder inflammation, abnormal milk and systemic signs such as fever, increased heart rate, dehydration, weakness and depression.

Clinical mastisis may be *acute*, where the disease flares up relatively suddenly in a formerly healthy cow; these cases may be further defined as *per-acute*, where the rapid onset of severe inflammation, pain and systemic symptoms results in a severely ill cow within a short period of time, or *sub-acute* mastitis, the most frequently seen instance of the disease, where the few symptoms tend to be mild inflammation in the udder and visible changes to the milk, such as small clots.

Long-term recurring persistent cases of the disease are termed *chronic*. These cases may show few symptoms between repeated occasional flare-ups of the disease where symptoms are visible and can continue over periods of several months. Often with chronic mastitis, irreversible damage is caused to the udder from the repeated clinical occurrences of the illness, and often these cows are culled.

The most common way to detect clinical mastitis is stripping a few squirts of milk into a strip cup at the beginning of milking to check for abnormalities such as clots and flakes.

Visual observation and palpating the udder for signs of inflammation can also help identify clinical mastitis but should not substitute for stripping. (NMC, 2013).

Just as like mastitis in general, clinical mastitis may have various pathogenic causes like Staphylococcus aureus, Escherichia coli, Staphylococcus epidermidis, Streptococcus spp, or Klebsiella spp (Sumathi, et al., 2008). However, the occurrence of various strains may have wide regional and seasonal variation.

The emergence of clinical mastitis in herds may be strongly influenced by specific management strategies in the dry period. Significant factor is the somatic cell count on the animals' side. Even one somatic cell count that is higher than 200.000 cells/mL in the 3 months before drying off can have significant influence. A series of farm management factors related to hygiene were also identified that can be in connection with appearance of mastitis. Among these were the administration of dry cow treatments and management of the early and late dry-period accommodation and calving areas. Vaccination with a leptospirosis vaccine and selection of dry-cow treatments for individual cows within a herd rather than for the herd as a whole had also positive effect. Further management tools were the routine body condition scoring of cows at drying off, and a pasture rotation policy of grazing dry cows for a maximum of two weeks before a non-grazed period of four weeks. (Green, et al., 2007)

3.1.2. Subclinical Mastitis

Subclinical mastitis doesn't cause any symptoms which are visible to the naked eye in either the milk or changes to the other udder. The most prevalent sign is an increase in somatic cell count (SCC). SCC is seen to effect the lactation and reproductive performance of the cow.

Subclinical mastitis also jeopardizes milk quality, preventing dairy producers from getting valuable SCC premiums. In addition, it has been shown to increase the number of days open, meaning that producers will have to pay for additional services. Research presented at the 2015 National Mastitis Council Annual Meeting concluded the cost of subclinical mastitis often is greater than that of clinical mastitis. With an estimated cost to the U. S. dairy industry of more than $1 billion per year. A white blood cell differential is used to detect udder infections before visible symptoms occur, allowing dairy producers to take action and prevent losses (AAD, 2017).

Many incidences of mastitis are directly indicated only by a high individual cow somatic cell count through milk recording schemes or via California Mastitis Testing. Subclinical infection is more likely to be caused by contagious pathogens. The presence of a causative pathogen is done via a bacteriological culture and indirect indications of subclinical mastitis can be given via electro-conductivity testing of milk, which is performed in an automated form in many modern parlours and robotic milking systems.

Milk, fat, and protein loss due to a new subclinical mastitis case may be economically important, a study was carried out in the Netherlands to show the cost of subclinical mastitis. The loss was estimated based on test-day (TD) cow records collected over a 1-yr period from 400 randomly selected Dutch dairy herds. The production (milk, fat, or protein) losses were modelled as the difference between the actual and predicted production (milk, fat, or protein) at the TD of new subclinical mastitis, for 4,382 cow records, and 2,545 cow records after dilution correction. Primiparous cows were predicted to lose 0.31 (0.25–0.37) and 0.28 (0.20–0.35) kg of milk/d at an SCC of 200,000 cells/mL, for unadjusted and adjusted low SCC, respectively. For the same SCC increase, multiparous cows were predicted to lose 0.58 (0.54–0.62) and 0.50 (0.44–0.56) kg of milk/d, respectively. Moreover, it was found that the greater the SCC increase above 100,000 cells/mL, the greater the production losses. (Halasa, et al., 2009)

The use of intramammary antibiotics to treat cows subclinically infected with Strep. agalactiae is usually successful and results in increased production and dramatic decreases in bulk tank SCC. In contrast, it is not considered cost-effective to treat most cows that are chronically infected with Staph. aureus because cure rates during lactation are generally quite poor. The difference in these therapeutic outcomes is thought to be related to differences in the site of infection. Some pathogens (Strep ag, CNS, E.coli etc.) infect superficial surfaces (such as the epithelial surface of the ducts). Other pathogens (Staph aureus, Strep uberis etc.) are invasive and it is more difficult to achieve a therapeutic concentration of antimicrobial at the site of infection (Petersson-Wolfe & Currin, 2012).

3.2. Pathogens concerned

A series of bacteria may be the cause behind mastitis cases. In a study dealing with the incidence rate of clinical mastitis on Canadian farms, authors concluded that Staphylococcus aureus, Escherichia coli, Streptococcus uberis, and coagulase-negative staphylococci were most frequently isolated mastitis pathogens. In total, 106 dairy farms

from 10 provinces participated in the study for a period of one year, recording 3.149 cases of clinical mastitis (Olde Riekerink, et al., 2008).

Subclinical mastitis may have similar pathogen background, however, local and temporal changes may occur. Researchers in Sweden described that Staphylococcus (S) aureus (19%) and coagulase-negative staphylococci (CNS; 16%) followed by Streptococcus (Str) dysgalactiae (9%), Str. uberis (8%), Escherichia (E.) coli (2.9%), and Streptococcus spp. (1.9%) were the most common isolates of 590 bacteriological diagnoses they studied. In their findings negative samples constituted 22% and 18% of the diagnoses, belonging to no growth or contamination respectively. The authors warn about the increased risk of finding S. aureus, Str. uberis or Str. dysgalactiae in milk samples from chronically infected cows compared to findings in milk samples from newly infected cows (Persson, et al., 2011)

3.2.1. Streptococcus

Streptococcus uberis is Gram-positive, with a cell wall structure similar to Staphylococcus spp., as well as streptococci such as S. agalactiae and S. dysgalactiae. S. uberis is the most common Streptococcus species isolated from cases of mastitis. Streptococcus uberis is the cause of 70% of clinical mastisits (Lopez-Benavides, et al., 2007)

New infections can occur at any time during lactation and may also occur during the dry period. However, the risk for new infection with S. uberis has been reported greatest during the early dry period. Following milk cessation, cows do not experience the daily flushing of the gland and are at an increased risk for mastitis in the early dry period. Cows in early lactation are also at increased risk for new infections due to the increased stress and immune suppression associated with the postpartum period. Cows with high milk production are not at greater risk than cows with low milk production (Petersson-Wolfe & Currin, 2012).

3.2.2. E.coli

E. coli and other coliform bacteria are found in high concentration in organic matter, such as bedding and manure. Udders become infected with coliform bacteria through contact with organic matter in the environment or during milking.

Once inside the udder, E. coli multiply rapidly, causing an influx of inflammatory cells. As neutrophils ingest and kill bacteria, endotoxin (LPS) is released and along with other inflammatory mediators causes severe local inflammation. This inflammatory response is

characterized by increased vascular permeability, changes in milk composition and damage to the mammary epithelial cells – all of which may result in the characteristic watery or serous milk secretion.

Several studies have found that undifferentiated cases of mild or moderate coliform mastitis have a high rate of spontaneous cure, meaning that the cow's immune system is able to clear the infection without antimicrobial therapy (UoM VDL, 2014).

Escherichia coli causes inflammation of the mammary gland in dairy cows around parturition and during early lactation with striking local and sometimes severe systemic clinical symptoms. This disease affects many high producing cows in dairy herds and may cause several cases of death per year in the most severe cases. It is well known that bacterial, cow and environmental factors are interdependent and influence mastitis susceptibility. Many studies, executed during the last decade, indicate that the severity of E. coli mastitis is mainly determined by cow factors rather than by E. coli pathogenicity. During E. coli mastitis, the host defence status is a cardinal factor determining the outcome of the disease. Today, we know that the neutrophil is a key factor in the cows' defence against intramammary infection with E. coli. Effective elimination of the pathogen by neutrophils is important for the resolution of infection and the outcome of E. coli mastitis. (Burvenich, et al., 2003)

3.2.3. *Staphylococcus*

Mastitis caused by Staphylococcus aureus (S. aureus*)* bacteria is extremely difficult to control by treatment alone. Successful control is gained only through prevention of new infections and cow culling. S. aureus organisms colonize abnormal teat ends or teat lesions. Milkers' hands, wash cloths, teat cup liners, and flies are ways in which the infection can be spread from cow to cow. The organisms probably penetrate the teat canal during milking. Irregular vacuum fluctuations impact milk droplets and bacteria against the teat end with sufficient force to cause teat canal penetration and possible development of new infection. Infected cows must either be culled, segregated from the milking herd and milked last or milked with separate milking units, or teat cup liners must be rinsed and sanitized after milking infected cows.

One of the most common types of chronic mastitis is caused by the bacteria, Staphylococcus aureus. Often, it is subclinical, where there is neither abnormal

milk nor detectable change in the udder, but somatic cell count has increased. Some cows may flare-up with clinical mastitis, especially after calving (Jones, et al., 1998).

Subclinical mastitis caused by intramammary infections with coagulase-negative staphylococci is common in dairy cows and may cause herd problems. Control of coagulase-negative staphylococci mastitis is complicated by the fact that coagulase-negative staphylococci contain a large number of different species. Persistent intramammary infections were common in quarters infected with Staphylococcus chromogens, Staphylococcus epidermidis, and Staphylococcus simulans. The results did not indicate differences between these coagulase-negative staphylococci species in their association with daily milk production, cow milk somatic cell count, and month of lactation in cows with subclinical mastitis. In cows with subclinical mastitis, S. epidermidis intramammary infections were mainly found in multiparous cows, whereas S. chromogens intramammary infections were mainly found in primiparous cows. (Thorberg, et al., 2009)
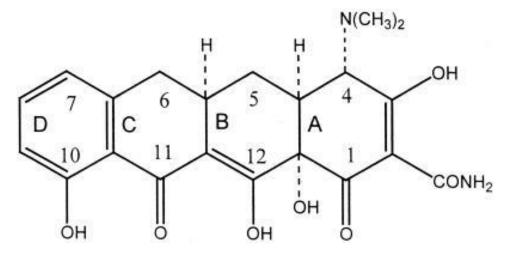
3.3. Antibiotics

For my study I have chosen two very commonly used intra mammary antibiotics, tetracycline and neomycin.

3.3.1. Tetracycline

Tetracycline's were discovered in the 1940s and exhibited activity against a wide range of microorganisms including gram-positive and gram-negative bacteria, chlamydiae, mycoplasmas, rickettsiae, and protozoan parasites. They have also been used extensively in the prophylaxis and therapy of human and animal infections and also at sub therapeutic levels in animal feed as growth promoters. The tetracycline's, which were discovered in the 1940s, are a family of antibiotics that inhibit protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor site. Tetracycline's are broad-spectrum agents, exhibiting activity against a wide range of gram-positive and gram-negative bacteria, atypical organisms such as chlamydiae, mycoplasmas, and rickettsiae, and protozoan parasites. The favourable antimicrobial properties of these agents and the absence of major adverse side effects has led to their extensive use in the therapy of human and animal infections. They are also used prophylactically for the prevention of malaria caused by mefloquine-resistant Plasmodium falciparum.

Tetracycline molecules comprise a linear fused tetracyclic nucleus to which a variety of functional groups are attached. The simplest tetracycline to display detectable antibacterial activity is 6-deoxy-6-demethyltetracycline and so this structure may be regarded as the minimum pharmacophore

1. Figure - Chemical structure of tetracycline (Eisenhart & Disso, 2012)

It is well established that tetracycline's inhibit bacterial protein synthesis by preventing the association of aminoacyl-tRNA with the bacterial ribosome. Therefore, to interact with their targets these molecules need to traverse one or more membrane systems depending on whether the susceptible organism is gram positive or gram negative. Hence, a discussion of the mode of action of tetracycline's requires consideration of uptake and ribosomal binding mechanisms. Also pertinent to this discussion are explanations of the joint antibacterial-antiprotozoal activity of the tetracycline's and the microbial selectivity of the class as a whole. Most of these issues have been considered at length in recent years, so the focus here will be on new information (Chopra & Roberts, 2001).
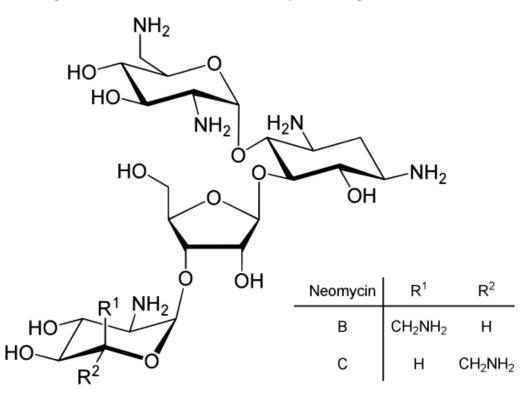
3.3.2. Neomycin

Neomycin is an aminoglycoside antibiotic found in many topical medications such as creams, ointments, and eye drops. The discovery of neomycin dates back to 1949. It was discovered in the lab of Selman Waksman. Neomycin belongs to aminoglycoside class of antibiotics that contain two or more amino sugars connected by glycosidic bonds. Neomycin is active against a large variety of bacteria, including Gram-positive and Gram-negative, as well as acid-fast, forms. It is active alike against streptomycin-sensitive and streptomycin resistant strains of bacteria, including those of M. tuberculosis var. hominis.

It is not active against fungi. Neomycin is not only bacteriostatic but also strongly bactericidal. It does not readily allow development of resistant strains of bacteria among the sensitive forms (Waksman, et al., 1949).

Neomycin inhibits the binding of Tat-derived peptides to the trans-activating region (TAR) of HIV-1 RNA. Kinetic studies reveal that neomycin acts as a non-competitive inhibitor that can bind to the Tat−TAR complex and increase the rate constant (koff) for dissociation of the peptide from the RNA. Neomycin effects a conformational change in the structure of TAR that can be detected by circular dichroism spectroscopy. The increase in ellipticity measured at 265 nm upon binding of the aminoglycoside is opposite to the decrease seen when Tat peptides bind to the RNA. Thus, the structural transition induced by neomycin is apparently incompatible with the binding of Tat and underlies the inhibitory action of the antibiotic. (Wang, et al., 1998)

2. Figure - Chemical structure of neomycin (Jiang, et al., 1999)

Neomycin	R^1	R^2
B	CH_2NH_2	H
C	H	CH_2NH_2

3.3.3. Mastijet

Mastijet is the name of the formulation used in the study. It is an oil suspension targeted for lactating cows. It covers both gram positive and gram negative bacteria and is specifically designed for bacteria sensitive to tetracycline and neomycin. The dosage is one

intra mammary infusion every 12 hours per infected quarter with a maximum for four treatments. The withdrawal periods for meat is twenty-one days and for milk is six milking (three days). It should be stored at 25°C and not frozen. Mastijet should not be administered to patients with a known allergy to any of the contents.

Each 8 gram injector contains: tetracycline: 200 mg; neomycin: 250 mg; prednisolone: 10 mg (MSD Animal Health, 2009-2017).

3. Figure - Mastijet syringes (MSD Animal Health, 2009-2017)

3.4. Yoghurt: uses and benefits

Yoghurt is considered as a nutrient dense food that contains essential nutrients such as protein, vitamins and minerals necessary for growth. Consumption of dairy products such as yoghurt helps to improve the overall quality of the diet. Lactose is the main carbohydrate found in milk which is a disaccharide composes of one molecule of glucose and galactose. Lactose is broken down to its simple sugars due to the action of the enzyme, lactase inside the gut. Inadequacy of secretion or interferences to the digestion process of lactase may pass undigested lactose into the large intestine which will then be fermented by colonic microflora that results gastrointestinal symptoms such as flatulence, diarrhoea and abdominal pain. This phenomenon is called as the lactose intolerance. It has been reported that the lactose intolerance is associated with low calcium intake and bone mineral density most probably unnecessary exclusion of milk and dairy products from the

diet. Therefore, it can be concluded that yoghurt is effective for the individuals with lactose intolerance to attain all the benefits of milk products without causing discomforts associated with hypolactasia (Weerathilake, et al., 2014).

In addition to involatile components, volatile compounds also contribute to the aroma and flavour of yogurt to a significant extent. Most flavour compounds in yogurt are produced by the microbiological transformations of lactose and citrate and the lipolysis of milk fat. Over one hundred different volatile compounds are found in yogurt at low, even trace concentrations, like carbonyl compounds, alcohols, acids, esters, hydrocarbons, aromatic compounds, sulfur-containing compounds, and heterocyclic compounds. Besides lactic acid several other components contribute to the characteristic aroma and flavour of yogurt, acetaldehyde, acetone, and iso-butanone among them. Extended storage of yogurt causes off-flavour development, mainly due to the production of undesired aldehydes and fatty acids during lipid oxidation (Cheng, 2010).

It is generally accepted that the optimum balance in the intestinal microflora is associated with good nutrition and health. Further, Lactobacilli and Bifidobacterium are known to be the primary microbial strains associated with this balance. Available research findings suggest that maintaining favourable microbial profile through regular consumption of bio-yoghurt results numerous therapeutic benefits. Yoghurt acts as a probiotic carrier food that is considered as an easy food to incorporate probiotics which results high probiotic viability. Bio-yoghurt is considered to be an ideal source for the delivery of viable probiotic strains, L. acidophilus and Bifidobacterium bifidum which are the most common probiotics used in the dairy industry. However, in order to attain the probiotic effect, it is reported the need of consuming adequate amounts of viable probiotic cells regularly which is known as the therapeutic minimum. Yoghurt consumption is also reported to be effective in cytokine production, T-cell function and natural killer-cell activity, and thereby result an overall immunological enhancement. (Weerathilake, et al., 2014)

In addition to its positive nutritional effects, consuming yoghurt may have beneficial impact directly on human health, too. The discovery of yoghurt-type fermented dairy products by the Western societies came from the observations of the Ukrainian scientist E. Metchnikov, who described the significant variances between the lifespan of Caucasian and European shepherds and connected this difference to the consumption of fermented dairy product. He was also the first identifying the Lactobacillus bulgaricus and Streptococcus thermophiles strains (Baglio, 2014). Later studies proved positive effects of

probiotics present in yoghurt cultures in treating metabolic disease, lowering serum cholesterol levels, lowering blood pressure via inhibiting angiotensin-converting enzyme, or even improving oral hygiene and therefore increasing the efficiency of dental treatments (Jones, et al., 2012) (Donkor, et al., 2007) (Kavaloglu Cildir, et al., 2009)
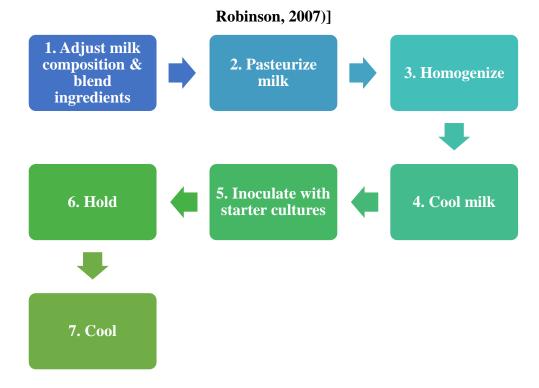
3.5. Yoghurt making process

Yoghurt is a fermented milk product that contains the characteristic bacterial cultures Lactobacillus bulgaricus and Streptococcus thermophiles. All yoghurt must contain at least 8.25% solids not fat. Full fat yoghurt must contain not less than 3.25% milk fat, low-fat yoghurt not more than 2% milk fat, and non-fat yoghurt less than 0.5% milk (Tamime & Robinson, 2007).

The usual pathway of fermentation in yoghurt making is homolactic fermentation. This process takes place in two steps. In the first step glucose is transformed into pyruvic acid, in the second one this is reduced to lactic acid. At the same time it is important to note that the processes of yoghurt making differ in two distinct features from the general homolactic fermentation processes. Firstly, the fermentation is carried out by two bacteria strains mentioned above. Secondly, these lactic acid bacteria are able to produce significant amounts of organic acids by chemically converting lactose without competition. On the contrary, Lactobacillus bulgaricus and Streptococcus thermophiles can act synergic ally, and this renders yoghurt making a very specific process. Not like other opportunistic associations, the synergic interaction in this case is very efficient due to the intensive production of lactic acid, acetaldehyde and polysaccharides (Castillo Martinez, et al., 2013) (Baglio, 2014)

The main ingredient in yoghurt is milk. The type of milk used depends on the type of yoghurt – whole milk for full fat yoghurt, low fat milk for low-fat yoghurt, and skim milk for non-fat yoghurt. Other dairy ingredients are allowed in yoghurt to adjust the composition, such as cream to adjust the fat content, and non-fat dry milk to adjust the solids content. Stabilizers may also be used in yoghurt to improve the body and texture by increasing firmness, preventing separation of the whey (syneresis), and helping to keep the fruit uniformly mixed in the yoghurt.

The main starter cultures in yoghurt are Lactobacillus bulgaricus and Streptococcus thermophiles. The function of the starter cultures is to ferment lactose (milk sugar) to produce lactic acid. The increase in lactic acid decreases pH and causes the milk to clot, or

form the soft gel that is characteristic of yoghurt. The fermentation of lactose also produces the flavour compounds that are characteristic of yoghurt.

4. Figure - General yoghurt processing steps [own compilation by (Tamime & Robinson, 2007)]

Milk composition may be adjusted to achieve the desired fat and solids content. Often dry milk is added to increase the amount of whey protein to provide a desirable texture. Ingredients such as stabilizers are added at this time.

The milk mixture is pasteurized at 85°C for 30 minutes or at 95°C for 10 minutes. A high heat treatment is used to denature the whey (serum) proteins. This allows the proteins to form a more stable gel, which prevents separation of the water during storage. The high heat treatment also further reduces the number of spoilage organisms in the milk to provide a better environment for the starter cultures to grow. Yoghurt is pasteurized before the starter cultures are added to ensure that the cultures remain active in the yoghurt after fermentation to act as probiotics; if the yoghurt is pasteurized after fermentation the cultures will be inactivated.

The blend is homogenized (13.8 to 17.2 MPa) to mix all ingredients thoroughly and improve yoghurt consistency. The milk is cooled to 42°C to bring the yoghurt to the ideal growth temperature for the starter culture. The starter cultures are mixed into the cooled milk. The milk is held at 42°C until a pH 4.5 is reached. This allows the fermentation to progress to form a soft gel and the characteristic flavour of yoghurt. This process can take

several hours. The yoghurt is cooled to 7°C to stop the fermentation process (Tamime & Robinson, 2007).

In addition to the main raw materials as milk and yoghurt culture, several additives are used during yoghurt making processes. These additives - as inulin, for example - may contribute to the texture and microstructure of yoghurt, its rheological and sensory values. These are used to influence the physical-chemical and sensory properties of yoghurt and thus contribute to the development of various dairy product lines (Guggisberg, et al., 2009).

4. Materials and methods

The pasteurization and yoghurt making were carried out in the Food Technology Laboratory of Food Hygiene Department of the university. Sample processing and instrumental measurements were carried out in the Food Toxicological laboratory of the same department.

4.1. Chemicals and reagents

Neomycin trisulfate salt hydrate and tetracycline hydrochloride were used as reference standards and also for spiking (i.e. artificially contaminating) the milk to be pasteurized and processed into yoghurt. Penicillin-V potassium salt was used as internal standard (ISTD) for the LC-MS/MS measurements. Stock solutions of standards at 1 $mg \cdot mL^{-1}$ concentration were prepared in 1:9 acetonitrile/water mixture. Working solutions of 100,000 $ng \cdot mL^{-1}$, 10,000 $ng \cdot mL^{-1}$, 1,000 $ng \cdot mL^{-1}$, and 200 $ng \cdot mL^{-1}$ of each individual antibiotic, as well as, 100,000 $ng \cdot mL^{-1}$ from penicillin-V (ISTD) were prepared daily by diluting the stock solutions with 1:9 acetonitrile/water mixture.

4.2. Method validation

Before starting the trials validation of the food analytical method was carried out in line with the requirements by the corresponding EU legislation and scientific guidelines (Eurpean Commission, 2002) (EMEA, 2011). Blank raw yoghurt samples were spiked with the calculated level of the given antibiotics and subjected to the normal sample preparation method to be described below. Internal standard (penicillin-V) was added to 1,000 μL of raw milk to obtain 1,500 $ng \cdot mL^{-1}$ final concentration before sample processing. As the validation of the analytical method, specificity/selectivity, linearity, limit of detection (LOD or decision limit - CCα), limit of quantitation (LOQ or detection capability – CCβ), within and between-run accuracy (precision and trueness) and recovery after sample preparation were determined. The analytical method used for this study met the requirements of validation reliability set in the guidelines referred above.

4.3. Artificial contamination of milk

Around 50 litres of raw milk was purchased for each trial from the local market. In average, it contained 3.97 % fat, 3.21% protein, 4.75 % lactose and 8.65 % non-fat dry matter; its pH was 6.69. (Milk quality data are from the Hungarian Dairy Research Institute, Raw Milk Laboratory and were provided by the producer.)

48 litres of milk was artificially contaminated ("spiked") with the desired amount of each antibiotic to obtain the required level of antibiotics. Then milk was homogenised manually and maintained at room temperature for approx. 20 minutes to allow the equilibration of the antibiotics with the milk matrix. The antibiotic levels of each trial were aimed to model the first, second and third days after medication, respectively.

1. Table - Concentrations of the studied antibiotics in the spiked milk

Compound	Concentration (ng/mL; mg/L; ppb)		
	H(igh)	**M(edium)**	**L(ow)**
tetracycline	1 800	350	150
neomycin	15 000	1 500	500

Before pasteurization 8 litres of milk was removed to be processed into dairy products without heat treatment. The remaining 40 litres went through pasteurization and become raw material of further dairy product preparation after it. Samples for antibiotic content determination were taken both before and after the heat treatment.

4.4. Pasteurization

In each trial 40 litres of raw milk was pasteurized on the pilot-scale pasteurizer system available in the Food Technology Laboratory of the Department of Food Hygiene. This system (developed by Agrometal Food-tech Kft.), equipped with plate heat exchanger is aimed at educational and research purposes and therefore its capacity and dimensions are lower (150 litre per hour) than of the machines used in the dairy industry. However, in other aspects is analogue with the commonly used pasteurizer systems. The milk was kept at 75 °C for 30 sec as it is also used in the dairy industry.

After pasteurisation the milk was cooled down and samples were taken from it. These samples were then homogenised in an ultrasonic bath for 2 minutes. After cooling the sample was divided into three sub-samples and the sample preparation procedure took place after adding the required amount of internal standard. The remaining part of the pasteurized milk served as raw material for yoghurt making.

4. Figure -Pasteurizer equipment in the Food Technology Laboratory of UoVM

4.5. Yoghurt making

The appropriate amount of yoghurt culture was added to ~50ml of milk (pasteurized or not-treated) after it was heated to 46 ℃ to dissolve the culture. This mixture was then added to the milk. After this the milk was then incubated for 3.5-4 hours at 46 ℃. I then refrigerated (6 ℃) the mixture for six hours.

4.5.1. Culture for yoghurt making

YoBiotik Natur yoghurt culture was the culture used in the study. YoBiotik Natur yoghurt culture with large amounts of probiotic bacteria. The product has a pleasant, yoghurt flavour and fragrance, hard and nice texture. Bulgarian type of yoghurt can be prepared by using this culture, due the original Bulgaricus bacteria present in it. It is rich in bacteria, what makes the product very healthy. It is characterized by mild suppurative acidity, therefore it maintains its excellent flavours and texture for a long time. One can make it fruity, muesli, drinking or Greek yoghurt. It shall be stored at +4 ° C (on the bottom, colder shelves or freezer of refrigerator) if stored in a deep freezer, place it at room temperature 1 hour before use.

Ingredients:

→ streptococcus thermophiles

→ lactobacillus delbrueckii subs. Bulgaricus

→ Bifidobacterium lactis HN019

→ inulin (prebiotic vegetable fiber)

Dosage: 1 capsule for 5 to 10 litres of milk is sufficient. (5 litres of milk makes thicker, 10 litres of milk makes thinner yoghurt) (Pozsa, 2016).

In this study thicker yoghurt was made from one litre of milk and dividing the content of culture capsules into five equal doses by measurements on analytical balance.
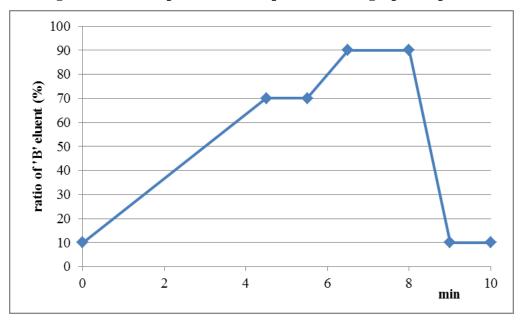
4.6. Sample preparation procedures

The milk and yoghurt samples were treated in the following way. 80 μL 10 % (v/v%) acetic acid was added to 1,000 μL milk or yoghurt sample. It was vortexed for 30 secs, and then 15 μL 1 M NaOH solution was added. The mixture was vortexed again for 15 secs, and then it was centrifuged at 12,000 rpm and 15 °C for 10 minutes. The supernatant was filtered through 0.22 μm syringe membrane filter and then analysed by LC-MS/MS.

4.7. Chromatographic analysis

Antibiotic content of the milk and yoghurt samples was analysed using a Shimadzu LCMS-8030 liquid chromatographic-mass spectrometry system consisting of a Shimadzu Nexera HPLC and a LCMS-8030 triple-quadrupole mass spectrometer. The chromatographic column was a Kinetex C_{18}, 100 x 4.6 mm ID (2.6 µm particle size) with a 4 x 2 mm C_{18} guard column.

For the gradient elution mobile phase 'A' consisted of 0.1 % (v/v%) formic acid in water, mobile phase 'B' was 0.1 % (v/v%) formic acid in acetonitrile. The flow rate was 0.6 mL/min; one chromatographic run lasted for 10 minutes. The gradient profile was as follows: t_0: B = 10 %; $t_{4.5}$: B = 70 %; $t_{5.5}$: B = 70 %; $t_{6.5}$: B = 90 %; t_8: B = 90 %; t_9: B = 10 % (where 't' refers to time in min, see figure 5). The autosampler was maintained at 10 °C, while the column at 30 °C. Injection volume was 15 μL. Equations of the calibration curves were calculated by the Shimadzu Lab Solutions software controlling the LC-MS/MS instrument, using the least squares method.

5. Figure - Gradient profile of the liquid chromatographic separation

Mass spectrometric circumstances were the following: electrospray ion source operated in positive ionisation mode (ESI+). Two multiple reaction monitoring (MRM) transitions were monitored for each compound. The interface voltage was 4.5 kV, interface temperature 350 °C, desolvation line temperature 300 °C, heat block temperature 450 °C, detector voltage 1.78 kV, nebulising gas (nitrogen) flow 3 L·min^{-1}, drying gas (also nitrogen) flow 15 L·min^{-1}. Collision gas was argon used at 230 kPa pressure in the collision cell.

5. Results and discussion

When looking at the results of the study, I took the three levels of artificial contamination as low, medium and high with a blank sample as a control. In Table 1, seen previously, I have broken down the nominal concentration into the concentrations for each of the antibiotics used. These are the theoretical values of what I added to the samples. In the "Milk" column you can find the values measured in the control (not heat-treated) and the heat treated milk samples. These number may be very close to the theoretical ones but may differ slightly, this may be because concentration of milk could occur or possibly due to precipitation. Similarly in the "Yoghurt" column you may find the values measured for the control (not heat-treated) and the heat treated samples. The pH of the yoghurts were between 4.2-4.33.

2. Table - Concentrations of contamination of each antibiotic measured in each sample

Concentration level	Heat treatment	Milk		Yoghurt	
		Measured (ng/mL)		Measured (ng/mL)	
		Neomycin	Tetracycline	Neomycin	Tetracycline
blank	control			<	<
	75 ℃			<	<
low	control	516	174	390	165
	75 ℃	485	165	376	149
medium	control	1,441	348	937	200
	75 ℃	1,238	334	778	176
high	control	15,256	1,716	5,090	1,005
	75 ℃	12,821	1,547	2,344	726

(Concentrations in ng/mL)

At low concentrations 516 ng/mL neomycin and 174 ng/mL tetracycline were measured in the untreated milk while 485 ng/mL neomycin and 165 ng/mL in the heat treated one. The control yoghurt (made from not heat treated milk) in the low level trial contained 390 ng/mL neomycin and 165 ng/mL tetracycline. These values were 376 ng/mL and 149

ng/mL for neomycin and tetracycline, respectively, in the case of yoghurt made from the heat treated milk. In the medium level trial the control milk measured 1,441ng/mL of neomycin and 348 ng/mL tetracycline, the heat treated milk measured 1,238ng/mL neomycin and 334ng/mL tetracycline. In this trial the control yoghurt measured 937 ng/mL neomycin and 200 tetracycline. Analysis of the yoghurt made from the heat treated milk in this level resulted in 778 ng/mL of neomycin and 176 ng/mL of tetracycline. In the high level trial the control milk measured 15,256 ng/mL neomycin and 1,716ng/mL tetracycline. The milk after heat treatment measured 12,821 ng/mL neomycin and 1547 tetracycline. The control yoghurt contained 5,090 ng/mL neomycin and 1,005 ng/mL tetracycline. The yoghurt made from heat treated milk measured 2,344 ng/mL neomycin and 726 ng/mL tetracycline.

A representative LC-MS/MS chromatogram can be seen on the figure 6 showing the retention times of studied compounds, neomycin and tetracycline, as well as, that of penicillin-V used as internal standard for quantitation.

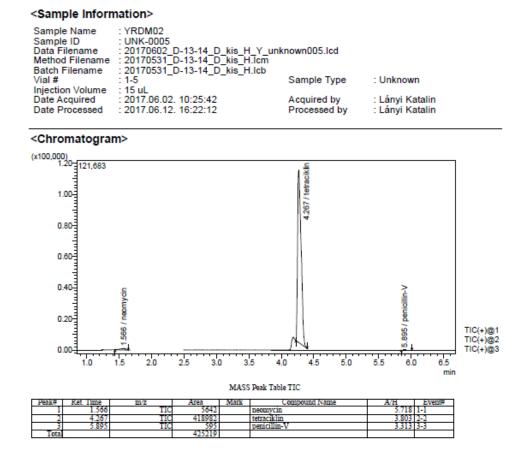## 6. Figure - A representative LC-MS/MS chromatogram

Table 3 summarises the losses in the concentration of antibiotic from the samples during the yoghurt making process. With the increase of starting antibiotic concentrations, more and more significant losses are observable.

3. Table - The difference between the concentration of antibiotics found in the milk and yoghurt
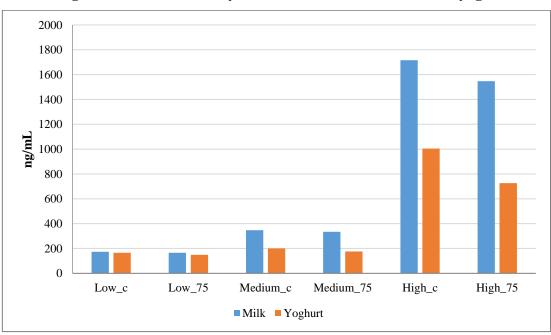
Concentration level	Milk-Yoghurt	
	Calculated	
	Neomycin	Tetracycline
blank		
low	126	9

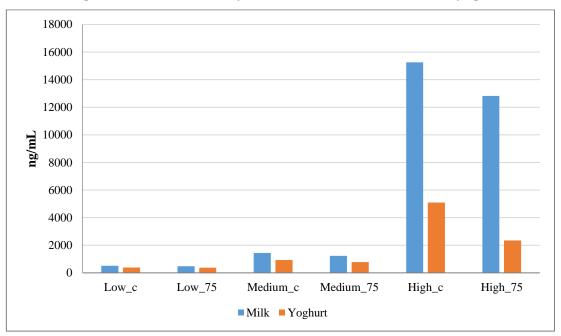|		
	109	16
medium	505	148
	461	158
high	10,165	711
	10,477	822

(Concentrations in ng/mL)

At low concentration the difference between the milk and yoghurt was 126 ng/mL of neomycin and 9 ng/mL of tetracycline in the control, while in the case heat treated milk it was 109 ng/mL neomycin and 16 ng/mL tetracycline. At medium contamination the difference was 505 ng/mL neomycin and 148 ng/mL tetracycline in the control while it was 461 ng/mL and 158 ng/mL in the heat treated samples. At the high level of contamination the difference was 10,165 ng/mL neomycin and 711 ng/mL tetracycline in the control. The difference in the heat treated samples was 10,477 ng/mL neomycin and 822 ng/mL tetracycline.

The figures 7 and 8 show there is far more of both antibiotics in the milk and yoghurt at the higher concentrations, than in the lower and medium concentrations. Maybe this could be due to the bacteria and antibiotic reaching equilibrium and all the bacteria being used up leaving a surplus of antibiotic at the higher concentration.

7. Figure - Measured tetracycline concentrations in milk and yoghurt

8. Figure - Measured neomycin concentrations in milk and yoghurt

Low_C: trial with low level of contamination (see section 4.3), without heat treatment

Low_75: trial with low level of contamination (see section 4.3), with heat treatment

Medium_C: trial with medium level of contamination (see section 4.3), without heat treatment

Medium_75: trial with medium level of contamination (see section 4.3), with heat treatment

High_C: trial with high level of contamination (see section 4.3), without heat treatment

High_75: trial with high level of contamination (see section 4.3), with heat treatment

As it can be seen from table 4 there was a noticeable increase in the amount of antibiotic going from the low concentration to the medium concentration of contamination. The neomycin increased from 24.40% and 22.40% in the control and heat treated sample respectively. The neomycin increased from 5.17% and 9.69% to 42.50% and 47.30% in the control and heat treated respectively. The percentage again increased going from the medium to the high. Neomycin going from 35.04% and 37.23% to 66.02% and 81.70% in the control and heat treated samples respectively. However, tetracycline decrease slightly in the control going from 42.5% to 41.43%. The heat treated samples did show an increase from 47.3% to 53.13% going from medium to high concentrations. These increases may be attributed to microbial useage and destruction by the antibiotic. Interestingly with this increase in the level of antibiotic there was a deterioration of the physical properties of the yoghurt with the low contamination more liquid and softer than the yoghurt and the medium not resembling yoghurt at all. The high contamination was in fact sour milk.

4. Table - Ratio of the antibiotic left in the yoghurt as percentage measured in milk

Concentration level	Heat treatment	Yoghurt/milk (%)	
		neomycin	tetracycline
blank	control		
	75 ℃		
low	control	24.40%	5.17%
	75 ℃	22.40%	9.69%
medium	control	35.04%	42.50%
	75 ℃	37.23%	47.30%
high	control	66.62%	41.43%
	75 ℃	81.70%	53.13%

5.1. Discussion

In all three levels of contamination the level of antibiotic decreased for both neomycin and tetracycline. This can be due to a number of factors. The first factor would be the heat sensitivity of the given compounds. As neomycin and tetracycline are both part of the group of antibiotics with intermediate heat stability they may show some heat degradation but shouldn't degrade completely. This may explain the lower values for the heat treated samples when compared to the control samples. Decomposition of tetracycline was seen by a study in America, which over different temperatures showed its degradation over time. (Eisenhart & Disso, 2012).

Heat degradation is not the only process which may contribute to the decrease in antibiotics. The antibiotics can undergo chemical decomposition due to acidic pH. Acidic pH was brought about by the yoghurt itself and the pH measurements taken ranged from 4.2-4.33. but a study has shown this is not a major factor, "tetracycline hydrochloride with a pH between 3 and 5 have been reported to be stable for 6 hours" and "suspensions of tetracycline hydrochloride with a pH between 4 and 7 are stable for at least 3 months." (Ali & Abdul-Jaleel, 2012). Neomycin is also considered stable in pH range from 2.0 to 9.0 (Simone & Popino, 1955). Taking this into account pH may not be a major factor in my study.

The contaminated samples did not resemble the control even at the low contamination it was of a more liquid consistency and softer. The medium consistency didn't really resemble yoghurt and the samples with high contamination was just sour milk.

5.2. Conclusions

This study has shown that the presence of antibiotic contamination has an effect on yoghurt making even at low levels of contamination. Given the change in the physical properties and the consumption of the antibiotic we could assume that the higher contamination of antibiotics had an influence on the yoghurt culture. It seems that with the higher concentrations of contamination the bacteria were not present long enough to culture yoghurt but still effected the pH and turned the milk sour.

I think it would have been a valuable part of the study if I had ran a microbial trial on the samples simultaneously with the study to see how long the bacteria were growing and maybe this information would have been useful to see how the pH was lowered but the yoghurt was not grown properly. In future studies carried out in this field I think it would be advisable to carry out a microbial trial of the sample or subject matter to get more detailed results and findings.

While there are other factors to the findings of my study like heat treatment and pH of the samples it can be assumed that most the inconsistencies with the culturing of the yoghurts was due to microbial usage of the antibiotics. The study has shown the importance of avoiding antibiotic contamination of milk on dairy farms and the importance of adhering to milk withdrawals of antibiotic drugs given to dairy cows. It has also highlighted the potential cost to dairy farmers and dairy processors if milk gets contaminated due to loss of viability of the milk.

6. Summary

The aim of this study was to see if the presence of antibiotics in milk had an effect on yoghurt making. Antibiotics are used in the dairy industry for the treatment and prevention of clinical and subclinical mastitis. The main bacterial pathogens concerned are streptococcus (mainly strep. Uberis), E. coli, and staphylococcus (mainly aureus). The antibiotics used in the study are neomycin and tetracycline, used in a formulation called Mastijet.

Before starting the trials validation of the food analytical method was carried out in line with the requirements by the corresponding EU legislation and scientific guidelines. Fifty litres of raw milk was purchased and 48 litres were spiked with the desired level of antibiotics. It was designed to resemble the first second and third days after treatment. It was then homogenised. Forty litres of the milk was then pasturized in the department of food hygiene lab keeping it at 75 °C for 30 sec as it is also used in the dairy industry. The appropriate amount of culture was then added to the milk to begin the yoghurt making process. Antibiotic content of the milk and yoghurt samples was analysed using a Shimadzu LCMS-8030 liquid chromatographic-mass spectrometry system consisting of a Shimadzu Nexera HPLC and a LCMS-8030 triple-quadrupole mass spectrometer.

The results of the study showed that the presence of antibiotics in milk has an effect on yoghurt making. Even on the low level of contamination there were some effects, there was a decrease in the presence of antibiotics due to the presence of the culture bacteria and it was physically different as it lost its consistency. At the highest level of contamination it had turned the milk sour.

This can all be link to the amount of antibiotics which were not present in the yoghurt but were seen in the milk. This difference was largely due to the microbial usage of the antibiotics, but also could be due to adjustment of pH and due to thermo-stability properties of the antibiotics.

The results of this study show the importance of food safety and the public health issues surrounding use of veterinary antibiotics on food producing animals. Its shows how residue of these drugs can affect the food processing industry at a financial cost as well as placing public health at risk. It shows the importance of following the withdrawal periods indicated with antibiotic drugs used on dairy cattle for the farmer, the processor and the consumer.

7. Bibliography

AAD, 2017. *Advanced Animal Diagnostics.* [Online]
Available at: http://www.qscoutlab.com/
[Hozzáférés dátuma: 17 09 2017].

AHDB, 2017. *AHDB Dairy.* [Online]
Available at: https://dairy.ahdb.org.uk/
[Hozzáférés dátuma: 08 10 2017].

Ali, M. & Abdul-Jaleel, M., 2012. Stability Study of Tetracycline Drug in Acidic and Alkaline Solutions by Colorimetric Method. *Journal of Chemical and Pharmaceutical Research,* 4(2), pp. 1319-1326.

Baglio, E., 2014. *Chemistry and technology of yoghurt fermentation.* Heidelberg: Springer Verlag.

Bar, D. és mtsai., 2008. The Cost of Generic Clinical Mastitis in Dairy Cows as Estimated by Using Dynamic Programming. *Journal of Dairy Science,* 91. kötet, p. 2205–2214.

Bord Bia, 2017. *Dairy - Domestic market.* [Online]
Available at: http://www.bordbiavantage.ie/market-information/sector-overviews/dairy/
[Hozzáférés dátuma: 12 10 2017].

Burvenich, C. és mtsai., 2003. Severity of E. coli mastitis is mainly determined by cow factors. *Veterinary Research,* 34. kötet, p. 521–564.

Castillo Martinez, F. A. és mtsai., 2013. Lactic acid properties, applications and production: A review. *Trends in Food Science & Technology,* 30. kötet, pp. 70-83.

Cheng, H., 2010. Volatile Flavor Compounds in Yogurt: A Review. *Critical Reviews in Food Science and Nutrition,* 50. kötet, p. 938–950.

Chopra, I. & Roberts, M., 2001. Tetracycline Antibiotics: Mode of Action, Applications, Molecular Biology, and Epidemiology of Bacterial Resistance. *Microbiology and Molecular Biology Reviews,* 65(2), p. 232–260.

CSO, 2016. *Statistics.* [Online]
Available at: http://www.cso.ie/en/statistics/
[Hozzáférés dátuma: 12 09 2017].

DAFM, 2016. *Annual Report 2016,* Dublin: Department of Agriculture, Food and the Marine (DAFM) of Ireland.

Donkor, O. és mtsai., 2007. ACE-inhibitory activity of probiotic yoghurt. *International Dairy Journal,* 17. kötet, p. 1321–1331.

Eisenhart, A. E. & Disso, N. M., 2012. *Thermostability Determination of Antibiotics at High Temperatures by Liquid Chromatography-Mass Spectrometry.* Weber State University, Ogden Utah, USA, National Conference On Undergraduate Research (NCUR), pp. 353-354.

EMEA, 2011. *Guideline on bioanalytical method validation.* London, United Kingdom: European Medicines Agency.

Eurpean Commission, 2002. *Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results.* Brussels, Belgium: the Commission of the European Communities.

Green, M., Bradley, A., Medley, G. & Browne, W., 2007. Cow, Farm, and Management Factors During the Dry Period that Determine the Rate of Clinical Mastitis After Calving. *Journal of Dairy Science,* 90. kötet, p. 3764–3776.

Guggisberg, D. és mtsai., 2009. Rheological, microstructural and sensory characterization of low-fat and whole milk set yoghurt as influenced by inulin addition. *International Dairy Journal,* 19. kötet, p. 107–115.

Halasa, T. és mtsai., 2009. Production loss due to new subclinical mastitis in Dutch dairy cows estimated with a test-day model. *Journal of Dairy Science,* 92. kötet, p. 99–606.

ICBF, 2017. *Irish Cattle Breeding Federation.* [Online]
Available at: https://www.icbf.com/wp/
[Hozzáférés dátuma: 27 09 2017].

Jiang, L. és mtsai., 1999. Saccharide–RNA recognition in a complex formed between neomycin B and an RNA aptamer. *Structure,* 7. kötet, p. 817–827.

Jones, G., Bailey Jr., T. & Roberson, J., 1998. *Staphylococcus Aureus Mastitis: Cause, Detection, and Control.* [Online]
Available at: http://www.thecattlesite.com/articles/679/staphylococcus-aureus-mastitis-

cause-detection-and-control/
[Hozzáférés dátuma: 12 10 2017].

Jones, M. L., Martoni, C. J., Parent, M. & Prakash, S., 2012. Cholesterol-lowering efficacy of a microencapsulated bile salt hydrolase-active Lactobacillus reuteri NCIMB 30242 yoghurt formulation in hypercholesterolaemic adults. *British Journal of Nutrition,* 107(1505–1513).

Kavaloglu Cildir, S. és mtsai., 2009. Reduction of salivary mutans streptococci in orthodontic patients during daily consumption of yoghurt containing probiotic bacteria. *European Journal of Orthodontics,* 31. kötet, p. 407–411.

Lopez-Benavides, M. és mtsai., 2007. Field observations on the variation of Streptococcus uberis populations in a pasture-based dairy farm. *Journal of Dairy Science,* 90(12), pp. 5558-66.

MSD Animal Health, 2009-2017. *MSD animal health.* [Online]
Available at: http://egypt.msd-animal-health.com/products/mastijet-fort/productdetails_124_121494.aspx
[Hozzáférés dátuma: 15 10 2017].

NMC, 2013. *What is the difference between clinical and subclinical mastitis?.* [Online]
Available at: https://www.el-lechero.com/digital-edition/2013/02/#?page=14
[Hozzáférés dátuma: 05 10 2017].

Olde Riekerink, R., Barkema, H., Kelton, D. & Scholl, D., 2008. Incidence Rate of Clinical Mastitis on Canadian Dairy Farms. *Journal of Dairy Science,* 91. kötet, p. 1366–1377.

Persson, Y., Nyman, A.-K. J. & Grönlund-Andersson, U., 2011. Etiology and antimicrobial susceptibility of udder pathogens from cases of subclinical mastitis in dairy cows in Sweden. *Acta Veterinaria Scandinavica,* 53. kötet, p. 36.

Petersson-Wolfe, C. S. & Currin, J., 2012. *Streptococcus uberis: A Practical Summary for Controlling Mastitis,* Petersburg, Virginia, USA: Virginia Cooperative Extension.

Pozsa, P., 2016. *Panni Sajtműhelye (Panni's Cheese Works).* [Online]
Available at: https://hazisajtkeszites.hu
[Hozzáférés dátuma: 09 10 2017].

Simone, R. M. & Popino, R. P., 1955. The stability of neomycin in several pharmaceutical preparations. *Journal of the American Pharmaceutical Association,* 44(5), pp. 275-80.

Sumathi, B., Veeregowda, B. & Gomes, A. R., 2008. Prevalence and antibiogram profile of bacterial isolates from clinical bovine mastitis. *Veterinary World,* 1(8), pp. 237-238.

Tamime, A. & Robinson, R., 2007. *Tamime and Robinson's Yoghurt: Science and Technology.* Cambridge, England: Woodhead Publishing Limited, part of the CRC Press.

Thorberg, B.-M., Danielsson-Tham, M.-L., Emanuelson, U. & Persson Waller, K., 2009. Bovine subclinical mastitis caused by different types of coagulase-negative staphylococci. *Journal of Dairy Science,* 92. kötet, p. 4962–4970.

UoM VDL, 2014. *Mastitis Pathogen Factsheet #1 - E. coli & Coliform Mastitis.* [Online]
Available at: https://www.vdl.umn.edu/services-fees/udder-health-mastitis/factsheets-resources
[Hozzáférés dátuma: 29 09 2017].

Waksman, S. A., Lechevalier, H. A. & Harris, D. A., 1949. Neomycin-Pproduction and Antibiotic Poperties. *Journal of Clinical Investigation,* 28(5), pp. 934-939.

Wang, S. és mtsai., 1998. Binding of Neomycin to the TAR Element of HIV-1 RNA Induces Dissociation of Tat Protein by an Allosteric Mechanism. *Biochemistry,* 37(16), p. 5549–5557.

Weerathilake, W., Rasika, D., Ruwanmali, J. & Munasinghe, M., 2014. The evolution, processing, varieties and health benefits of yoghurt. *International Journal of Scientific and Research Publications,* 4. kötet, pp. 8-9.

8. Acknowledgements

9. Appendices

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