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Oxytetracycline residues from spiked ovine milk to cheese: technological implications

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To my love and my family

LIST OF PAPERS

This Ph.D. thesis is based on the following papers:

I. Transfer of oxytetracycline from ovine spiked milk to whey and cheese

Cabizza R., Rubattu N., Salis S., Pes M., Comunian R., Paba A., Addis M., Testa M.C., Urgeghe P.P. (2017), **International Dairy Journal. 70; 12-17.**

II. Heat treatment of oxytetracycline spiked ovine milk: fate of the molecule and technological implications

Cabizza R., Rubattu N., Salis S., Pes M., Comunian R., Paba A., Daga E., Addis M., Testa M.C., Urgeghe P.P.

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1. Introduction

1.1. Ovine milk in history

The first farming systems were adopted by populations who inhabited the Fertile Crescent. The fertile crescent included ancient Mesopotamia and covered an area from the Siro-Palestinian-Lebanese region up to the Mediterranean side and southern region of Anatolia (Malossini, 2001). The farming systems were based on animal domestication, cereal and legume cultivation, dating back to 10000-9500 B.C. (Zeder, 2008), during the prehistoric Neolithic age (Stone Age).

The "Neolithic Revolution" in the Fertile Crescent was the first agricultural revolution in history and caused a change of habits in nomadic populations. The populations, who had previously engaged in hunting and wild harvesting, became devoted to breeding, farming and raising sheep. The agro-pastoral system resulting from the Neolithic Revolution changed people's eating habits from a diet based on animal hunting and harvesting wild fruits to a system based on agriculture and animal husbandry.

Sheep and goats were the first species to be domesticated. Initially the sheep were raised as a source of meat and wool (6000 B.C.), and only later, around 4000 B.C, they became important for milk (Chessa et al., 2009).

The domestication of sheep has had important effects on the morphology, behaviour and genetics of primitive breeds, leading to the creation and selection of breeds with better productive features, regarding wool, meat and milk (Rocha, Chen, & Beja-Pereira, 2011). The bronze statuettes representing the Shepherd

Warriors found in Sardinia (Fig. 1), which date back to the Bronze Age and the Nuragic Civilization (1800-1500 B.C.), portray the breeding of sheep, goats and cattle on the island, in that period.

Fig. 1 Sardinia shepherd-warrior Nuragic age, Museo Sanna (Sassari, Italy).

1.2. Production of ovine milk

The world production of milk in 2014 (FAOSTAT - Food and Agriculture Organization of the United Nations, 2017) was about 791 million tons per year. Almost the entire amount of milk derived from cow with an annual production of 652.3 million tons (82.5%), directly followed by minor species such as buffalo (13.6%, 107.7 million tons), goat (2.3%, 18.3 million tons), sheep (1.3%, 10.4 million tons), and camel (0.4%, 2.9 million tons).

In Asia, manufacturing sheep milk is widespread (4.85 million tons), particularly in China, which is the world leader in ovine milk production (1.53 million tons). In Europe 3.08 million tons of ovine milk is produced per year, and the main producers are all countries with a great tradition of milk derived products such as Greece (0.77 million tons), Romania (0.67 million tons), Spain (0.59 million

tons), Italy (0.37 million tons), and France (0.26 million tons) (FAOSTAT - Food and Agriculture Organization of the United Nations, 2017).

In 2016, in Italy the ovine population was 7.2 million (Istat, 2016), and the region with the highest number of sheep was Sardinia with 3.3 million, equal to 45.3% of Italian ovine livestock, followed by Sicily (10.1%), Lazio (9.7%), and Tuscany (5.8%) (Fig. 2).

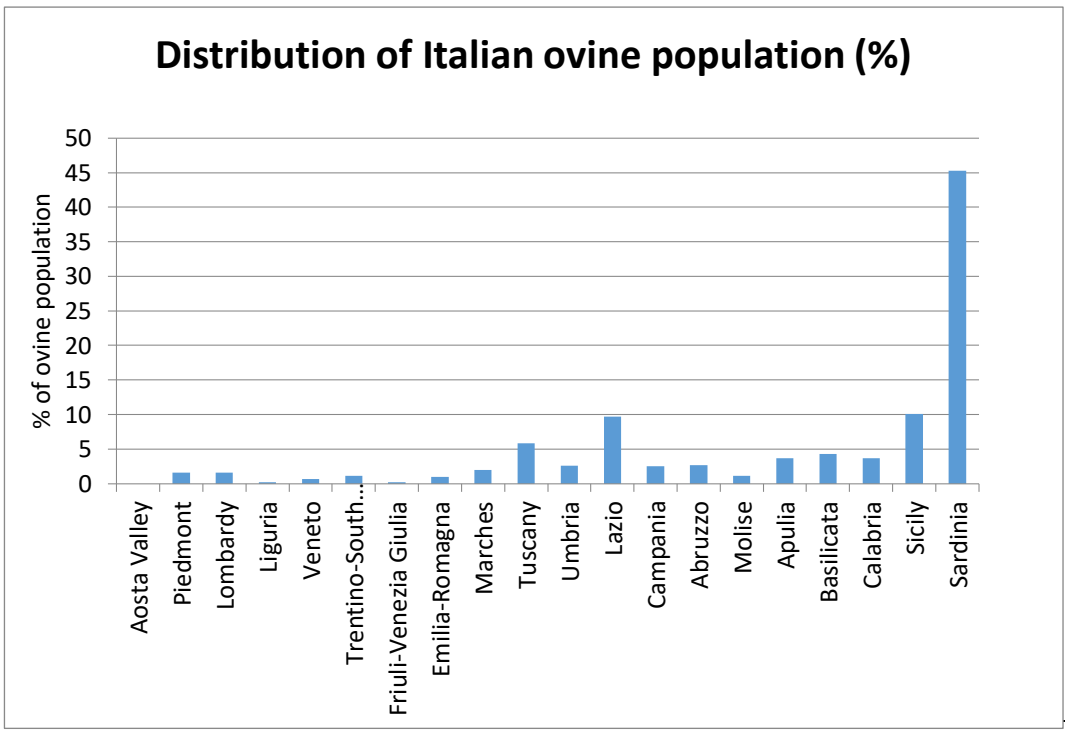

Fig. 2 Population of Italian ovine divided by regions (Istat, 2016).

In Sardinia the farming system is pasture-based, extensive and semi extensive based on the Sarda sheep breed, which is seasonally influenced by the Mediterranean climate (Vagnoni et al., 2015).

Ovine livestock have a great importance in Sardinia with high economic impact on the Island. In fact, the Sardinian sheep livestock farming system is composed of 12718 companies and 28542 farms with 16763 farmers, and Sardinia produces

about the 76% of Italian ovine milk (0.28 million tons per year) (Consorzio per la tutela del Formaggio Pecorino Romano, 2016). The Sardinian dairy system's target is to transform milk into cheese, in fact almost all the ovine milk produced in Sardinia is destined to cheese-making (Pirisi, Comunian, Urgeghe, & Scintu, 2011), while a minor amount is used to produce other milk derivatives (ricotta, yogurt, etc.). The Sardinian dairy industry is composed of 53 dairy cheese-making factories, 17 cooperatives societies and 2 milk collection centres (Istat, 2016). Sardinian sheep milk is mainly used to produce three PDO ovine cheeses (Protected Designation of Origin), such as Pecorino Romano, Pecorino Sardo, and Fiore Sardo (The Commission of the European communities, 1996, 2009; The European Commission, 2014).

1.3. Milk

Milk is one of the most interesting, complex and complete nutritional food, consumed from the first days of life until adulthood, in all parts of the world. It is a rich food where protein, lipids, sugar, vitamins and minerals (calcium) coexist and how infants receive the maternal antibodies as a defence against infections in the early days of life.

Milk is a heterogeneous biological liquid with high nutritional value consisting of three physical phases: an emulsion, a colloidal dispersion, and a solution. It is an opalescent white fluid substance showing a weakly acidic reaction, characterised by distinctive smell and a sweet taste.

The Regulation (EU) n. 1308/2013 of the European Parliament (The European Parliament and the Council of the European Union, 2013) establishes a common organisation of the markets in agricultural products and defines the term milk as follows: "*milk means exclusively the normal mammary secretion obtained from one or more milkings without either addition thereto or extraction therefrom"*. This Regulation also clarifies that: "*As regards milk, the animal species from which the milk originates shall be stated, if it is not bovine*".

1.3.1. Physico-chemical characteristic of ovine milk

Sheep milk has high protein and fat contents, almost twice the fat and casein content compared to cow milk, the highest casein content, compared to other species. This richness of components gives ovine milk a higher nutritional value (5932 kJ kg^{-1}) compared to other milks (Park, Juárez, Ramos, & Haenlein, 2007),

and makes it particularly suitable for cheese-making (Barlowska, Szwajkowska, Litwińczuk, & Król, 2011). The differences between sheep and other species, especially cow milk, are not only in composition (Table 1) (Chandan, Kilara, & Shah, 2008), but also in structure and organization of constituents. As for the milk of other species, the composition of sheep milk is influenced during the lactation period by genetic, physiological, dietary, environmental and technological factors (Alais, 2010).

Table 1
Proximate chemical composition of milk of mammals (%, w/w) (Chandan et al., 2008).

Mammal	Total solids	Fat	Total protein	Casein	Whey protein	Lactose	Ash
Cow	12.2	3.4	3.4	2.8	0.6	4.7	0.7
Buffalo	16.3	6.7	4.5	3.6	0.9	4.5	0.8
Goat	13.2	4.5	2.9	2.5	0.4	4.1	0.8
Sheep	19.3	7.3	5.5	4.6	0.9	4.8	1.0
Camel	13.6	4.5	3.6	2.7	0.9	5.0	0.7
Mare	11.2	1.9	2.5	1.3	1.2	6.2	0.5
Donkey	8.5	0.6	1.4	0.7	0.7	6.1	0.4

1.3.2. Carbohydrates

Lactose, a disaccharide derived from the condensation of galactose and glucose, is the major carbohydrate present in sheep milk (about 98% of total carbohydrate). It is important to maintain osmotic equilibrium during milk synthesis and the secretion phase. It is also important as a nutrient, and plays a key role in the intake of Vitamin D, and intestinal absorption of calcium, magnesium and phosphorus. Lactose is fundamental during the lactic acid fermentation performed by lactic acid bacteria, which converts lactose to lactic acid.

1.3.3. Lipids

Lipids are one of the most important fractions of sheep milk. In fact, ewe's milk is extremely high in fat content (4.5-7.5 %, w/w) and this characteristic has repercussions on the physical, nutritional, technological and sensorial profile of milk-derived products.

Fat is the most variable fraction of milk during the lactation cycle of sheep, and this strongly influences the cheese-making behaviour in absence of adequate fat to protein adjustments at the dairy plant. Triacylglycerols (TAG) are the main compounds of fat (98%), followed by simple lipids (diacylglycerols, monoacylglycerols, and cholesterol esters), complex lipids (phospholipids) and liposoluble compounds (sterols, cholesterol esters, hydrocarbons).

In sheep milk, the main fatty acids are saturated. Palmitic acid (C16:0; 25.9%) is the most present, followed by myristic acid (C14; 10.4%). The short-chain fatty acids are represented by butyric acid (C4:0; 3.5%), caproic acid (C6:0; 2.9%), caprylic acid (C8:0; 2.6%), and capric acid (C10:0; 7.8%). Oleic acid (C18:1; 21.1 %) linoleic acid (C18:2; 3.2%), conjugated linoleic acids (CLA; 1.1%), and linolenic acid (C18:3; 0.8%) are also markedly present (Park et al., 2007). Short and medium-chain fatty acids play a key role in the sensory profile of ovine cheeses, particularly those subject to a lipolytic ripening pathway (Fox, 2004).

Lipids in milk are organized in globules surrounded by a milk fat globule membrane (MFGM) which serves as an emulsion stabilizer, protecting them against the chemical and enzymatic degradation. MGFM is organised in a trilayer, which is composed of triacylglycerols, phospholipids, glycoproteins, enzymes and

cholesterol. The stability of MFGM is affected of physiological, physical and mechanical, and environmental factors. The fat globules dimension vary depending on the species, for example fat globules of sheep milk have a lower diameter (3.78 μm average size; with a range from 1 to 12 μm) compared to those of cow milk (3.95 μm average size; with a range from 1 to 16 μm) (Barlowska et al., 2011). The fat globule dimension has great influence during the technological and ripening process. In fact, the transformation of milk containing small fat globules (SFGs) allow to obtain a product with a higher retention of whey in cheese compared to cheese produced from milk containing large fat globules (LFGs). This difference is fundamental in order to obtain cheese with specific and desired characteristics such as soft or firm texture and influences the proteolysis during maturation (Barlowska et al., 2011). Milk fat dispersion varies depending on the species, and has consequence on creaming rate, rheology, technology and suitability for cheese-making (Barlowska et al., 2011), because of its influence on texture, flavour and physicochemical properties.

1.3.4. Proteins in sheep milk

Sheep milk shows high concentrations of proteins, which are very important both from a nutritional and technological point of view. The average amount of protein in sheep milk is higher than in cow milk (6.2% vs. 3.3%, respectively). The protein fraction is mainly composed of casein which represents 80% of the total, while whey protein constitutes 20%: this ratio greatly affects cheese yield. The casein fraction is formed of 4 proteins: αS1-casein (6.7%), αS2-casein (22.8%), β-

casein (61.6%), and *κ*-casein (8.9%) (Selvaggi, Laudadio, Dario, & Tufarelli, 2014). Caseins are in colloidal suspension in milk, and precipitate at pH 4.6 at room temperature. Caseins are associated with themselves and with calcium phosphate to form the casein micelle. Micelles are highly hydrated colloidal particles, with a diameter ranging from 150 to 200 nm (McSweeney & Fox, 2013). Many models have been elaborated to explain the structure and the behaviour of the casein micelle and each model has been proposed, based on more and more detailed scientific evidence. Dalgleish & Corredig (2012), Horne (2006) and McMahon & Oommen (2008) have reviewed many models. Some very important evidence is indicated below.

- Most of the properties of micelles are imputable to their surface properties, even being the inner portion involved in very important rearrangements during the post-coagulation phases;
- The binding of caseins with colloidal calcium phosphate (CCP) allow them to stabilize very high concentrations of calcium in the milk and is on the basis of the internal structure of the micelle;
- The micelle is highly hydrated (about 3.5 g of water per gram of protein), either on the surface and in the inner part. Its structure is open, and the β-casein can leave the micelle during cooling.

In the dual-binding model (Horne, 2006), the caseins link to the CCP to form small aggregates called *nanoclusters;* Nanoclusters seem to interact hydrophobically between themselves and with the αs and β-caseins. The k-casein is almost located on the surface of the micelles and stabilizes them as well as

limiting their growth. The amount of k-casein on the surface and the size of the micelle are inversely related with great consequences on rennet reactivity.

To explain the presence of water within the micelle, a recent model (D.G. Dalgleish, 2011) proposed the role of the β-caseins in stabilizing the formation of water micro-channels into the micelle, by interacting with the hydrophobic domains of the nanocluster.

1.3.5. Mineral fraction

Milk is a source of minerals, and sheep milk contains more minerals than human and cow milk. The mineral fraction is smaller than protein and lipid fractions. The most important minerals in milk are bicarbonates, chlorides, citrates of calcium, magnesium, potassium and sodium. Calcium, phosphate, citrate and magnesium are distributed between the soluble and colloidal phases. The interactions among minerals and milk proteins have consequences on the stability of milk and milk derivatives. Milk salts have an important effect on many proprieties of milk such as formation and stability of casein micelles, acid-bases buffer, nutritional role, colligative properties, protein stability during processing, emulsion stability and texture.

There are differences between the mineral composition of sheep and cow milk. Calcium (193 sheep vs 122 cow, mg 100 g^{-1} of milk), phosphate (158 sheep vs 119 cow, mg 100 g^{-1} of milk), magnesium (18 sheep vs 12 cow , mg 100 g^{-1} of milk) and zinc (0.57 sheep vs 0.53 cow, mg 100 g^{-1} of milk) are higher in sheep milk, while potassium (152 cow vs 136 sheep, mg 100 g^{-1} of milk), sodium (58

cow vs 44 sheep, mg 100 g^{-1} of milk) and manganese (0.02 cow vs 0.007 sheep, mg 100 g^{-1} of milk) have a higher concentration in cow milk (Park et al., 2007). Calcium is present in a significant amount as a complex with citrate and phosphate, and associated with casein micelles. It is also present as a free ion at a low concentration. Calcium and phosphate participate to form the casein micelle. In fact, calcium phosphate has a fundamental role in the stabilization of casein micelles. Micelles are rich in Phosphoserine (SerP) residues, negatively charged and able to bind calcium. Ca^{2+} binds two residues of SerP situated in two different micelles by a salt bridge. This network connects the sub-micelles to each other, which forms the inorganic structure of micelles.

Salts in colloidal form are in dynamic equilibrium with soluble form. The partition of calcium and phosphate between the colloidal and soluble phase is reported below (Masotti, Cattaneo, Rosi, & De Noni, 2011) :

- Colloidal Ca 67%; soluble Ca salt 21.5%; Ca ionic 11.5%;
- Colloidal P 53%; soluble P salt 47%, partition of P does not include the organic P amount present in casein micelle as SerP and in other molecules as phospholipids.

This equilibrium in milk is influenced by variations of pH, temperature, milk treatment and storage, which are able to compromise structure and stability of the micelles.

As the pH is reduced, CCP dissolves and is completely soluble at pH=4.9; the reverse occurs when the pH is increased. The solubility of calcium phosphate

decreases as the temperature is increased and soluble calcium phosphate is transferred to the colloidal phase.

Conversely, thermal treatment of milk causes a decrease of ionic calcium, and the subsequent reduced reactivity of milk during presamic coagulation.

1.3.6. Enzymes

Enzymes in milk are present both as indigenous and exogenous. Endogenous enzymes are naturally content in milk, while exogenous are derived from microorganisms and somatic cells. Many endogenous enzymes are important from a technological point of view and it is possible to classify them based on their function:

- Lipase and protease are involved in the alteration process of milk and derivatives but play a key role during the maturing of cheeses;
- Alkaline phosphatase and lactoperoxidase (LPO) are used to assay the thermal treatment of milk (pasteurisation and sterilisation) based on their sensitivity to heat. Some other enzymes can be used as markers of mild heat treatment (thermisation) such as α-L-fucosidase and γ-glutamyltransferase (De Noni, 2006; Piga et al., 2013);
- Lysozyme and lactoperoxidase (LPO) have antimicrobial activity;
- Catalase and acid phosphatase are used as indices of mastitic infection and good health of udder;

- Ribonuclease and lactoperoxidase (LPO) are a commercial source of enzymes.

1.3.7. Vitamins

Milk also contains small amounts of vitamins (<1%), with fundamental biological and nutritional roles. Vitamins are content in milk as water-soluble (A, D, E, and K) and fat-soluble vitamins (B and C). In their recent work Balthazar et al., (2017) provided a comprehensive comparison of vitamins present in the milk of different species. Sheep milk contains more vitamins than cow milk, especially vitamin A but a lower amount of carotene. In addition to their nutritional significance, vitamins are significant for other reasons: vitamin A (retinol) and carotenoids are responsible for the yellow-orange colour of fat-containing products made from cow's milk; vitamin E (tocopherols) is a potent antioxidant; vitamin C (ascorbic acid) is an antioxidant or pro-oxidant, depending on its concentration.

1.4. Cheese-making process

The cheese making process is one of the oldest methods known by man to conserve perishable food and to be able to eat rich food throughout the whole year. In the past it was a simple process not based on milk pre-treatment or use of any complicated technology. With time, man adopted technology to control each stage of the process to produce a variety of dairy products with different compositions, structure and sensory profiles.

It is possible to explain the process in a few main stages. To generalise there is the stage of coagulation, draining, moulding, acidification, salting and ripening. At the heart of the process is the transformation of milk into protein gel. This protein gel is obtained by the variation in stability of the casein micelles. This variation produces a change in their hydrophilic character, in fact casein micelles start to form a reticule through these hydrophobic interactions.

This effect can be obtained with different mechanisms, for example either through enzymes able to hydrolyse the bind 105-106 Phe Met (Phenylalanine - Methionine) of k-casein (enzymatic coagulation), or by decreasing pH through natural acidification caused by microorganism contaminating the milk or artificially added (*starter cultures)*, or by heat treatment in particular conditions.

In the matrix, the transition of the liquid-gel reticule in milk is the result of the balance between the hydrophobic interactions and the electrostatic repulsions. During the acid-induced gelation, the reduction of pH causes a reduction in the superficial charge of micelles, which provokes a decrease in the dissociated

carboxylic groups of caseins. This phenomenon induces a collapse of k-casein on the micelle.

Moreover, during the acidification the colloidal calcium content is progressively dissolved into the micelle. Hydrophobic interactions prevail against the electrostatic repulsion and form a weak gel not able to contract.

The enzymatic coagulation can be divided into three steps. The first step is the release of casein-glicomacropeptide (CGMP) by chymosin, which causes both a reduction of superficial charge and hydration of micelle. In the second step, micelles form an initial reticule from the hydrophobic interactions as well as the formation of bridges with calcium ions and colloidal calcium.

The presamic gel is a three-dimensional protein network that occupies the initial volume of milk, catching the other constituents. During the third step, the obtained gel is able to contract itself because of the exponential increase of interaction between the pseudo-micelles. This phenomenon causes the draining of whey from curd, called “spontaneous syneresis”.

The spontaneous syneresis of whey, in terms of time, is not compatible with dairy technology. For this reason, in cheese-making the syneresis is accelerated by mechanic operations performed on the gel, in order to obtain a curd able to aggregate and to retain a variable amount of whey. The first action needed is to break the clot in the vat, and during this phase, the main release of the whey is observed. The technology adopted during the breaking of the clot, and the subsequent phases of the process have a large effect on the amount and the composition of the whey and curd, the moisture of curd, and the recovery of the

milk's constituents of cheese, especially fat and protein. It is possible to classify the syneresis of cheese into three steps: primary in vats during draining between whey and curd; secondary during acidification, sweating and pressing phases; and tertiary stage during the salt phase.

Subsequently, the whey is drained from the vat and the curd is transferred into moulds. The selection of the mould considers the size and shape of the desired cheese. This phase is bound to the technology adopted for the cheese-making.

Successively, sweating and acidification phases are fundamental for the process. Cheeses are moved into rooms with controlled temperature and moisture. The level of which depends on the type of cheese. During this phase, the secondary syneresis starts and is helped by heat which is strictly correlated to the fermentative process of milk. Generally the room has a temperature in a range of 22-36°C with a relative humidity of about 90%. Temperature helps to aggregate the texture and to form the initial crust. The high relative humidity allows the cheese to maintain a soft surface and regulates the loss of weight. A correct acidification is fundamental to regulate the solubilisation of calcium from the coagulum and allowing a further draining of whey to obtain a cheese with desired characteristics. Additionally, it is an important phase in order to protect cheese from the development of undesirable microorganisms, which can be dangerous for human health and can cause defects on cheese due to undesired fermentations.

The subsequent phase of the process is salting. Salting contributes to the crust formations, the curd syneresis (tertiary stage), the flavour development and exerts protective role against the growth of undesirable non-starter microorganisms,

especially in cheese with high content of moisture. It is possible to perform this phase in different modes based on the type of technology adopted. Salting may be performed as follows: mixing dry salt with broken or milled curd at the end of manufacture; submerging cheese in brine; rubbing dry salt on the surface of the cheese; by combination of brine and rubbing salt.

During the ripening phase the cheese undergoes some important modifications, such as an additional dehydration and a complex pathway of biochemical reactions. The extent of these modifications depends on the adopted technology and is controlled at the ripening room by regulating the environment conditions (e.g temperature, humidity) and by surficial treatments of cheeses. The biochemical pathways of ripening result in specific characteristic of each type of cheese and have been well reviewed (McSweeney & Sousa, 2000). The main group of reactions involves the metabolism of the residual lactose and citrate, the proteolysis and the lipolysis.

1.5. The role of lactic acid bacteria during cheese-making

A starter culture can be defined as a pool of microorganisms added to a raw material able to drive the fermentation process in order to obtain fermented foods (Leroy & De Vuyst, 2004). To produce dairy fermented products (cheese, butter, yogurt, etc.), the action of starter lactic acid bacteria (SLAB) is fundamental to favour milk coagulation, confer desired sensory quality, guarantee safety, and reduce the appearance of defects in the final product. SLABs are a very important group of dairy microorganisms, able to decrease the pH and produce lactic acid from lactose. As well as this, they inhibit the growth of pathogens and spoilage bacteria, even by the production of other antimicrobial substances (Messens & De Vuyst, 2002). A good acidification of cheese enhances the drain of the curd which permits a desirable content of moisture in cheese (Santarelli et al., 2013). Furthermore, SLABs contribute to the development of the sensory profile (aroma compounds) and texture properties. The sensory profile is influenced by the fermentation of lactose and citrate, the degradation of proteins and fat (amino acids and free fatty acids metabolism), and by proteolytic and lipolytic activities.

Starter cultures can be divided on the basis of their optimum growth temperature, composition, and function. According to growth temperature it is possible to distinguish between mesophilic and thermophilic starter cultures. Mesophilic LAB cultures grow and produce lactic acid with at an optimal temperature of 30°C, while thermophilic LAB cultures have an optimum growth temperature at 42°C. The most used mesophilic species is the *Lactococcus lactis* subsp. *lactis*, while the most used thermophilic LAB cultures are *Streptococcus thermophilus*,

Lactobacillus delbueckii (subsp. *bulgaricus* and *lactis)*, and *Lactobacillus helveticus*.

Moreover, nonstarter lactic acid bacteria (NSLAB) contributes to the sensory profile producing aromatic compounds. Italian sheep milk cheeses are characterised by a great diversity of NSLAB microflora, which combined with the influence of technological and geographic aspects, increases the variably of cheeses (De Angelis et al., 2001; Mannu, Comunian, & Scintu, 2000).

1.6. Ovine cheeses

The Italian dairy sector is an important part of the country's economy, and in 2016 the volume of cheese exported was about 388 million tons, corresponding to a value of € 2.4 million (CLAL.it, 2017; Istat, 2017). The principal importer of Italian cheeses is the European Union (75%), especially France (21%), Germany (15%) and United Kingdom (9.1%). Italian cheeses are also appreciated in North America, in particular the United States of America, which imported 36000 tons (9.3%) in 2016, and it is the main importer of ovine cheeses (64%). Other countries that import Italian cheeses are Switzerland (5.1%), Japan (2.4%), South Korea (1%), and China (0.68%), which represent a rapidly growing market (CLAL.it, 2017).

Sheep milk is infrequently destined to direct for human consumption, but because of its high total solid, fat and protein content it is mainly used to produce cheese (Balthazar et al., 2017; Haenlein & Wendorff, 2006).

In many Mediterranean countries with a tradition of sheep farming, many types of ovine cheeses are produced, and most of them are recognised as Protected Designation of Origin (PDO) by the European Union.

At present 632 PDOs are recognized by the European Union and Italy is the country with the highest number of the PDO products (167) (UE, 2017). Actually, 50 Italian cheeses have the PDO trademark followed by France (45), Spain (26), Greece (21), and Portugal (11). The total contribution of ovine PDO cheeses is very relevant. In fact, 18 out of 50 (36%) of the Italian PDO cheeses are produced from ewe milk only (11) or from a mix of milk deriving from different species

including ovine milk (7). This underlines its importance among Italian dairy production.

Sardinia produces three PDO cheeses from ovine milk, Pecorino Romano, Pecorino Sardo, and Fiore Sardo (Table 2, 3) (Pirisi et al., 2011; The Commission of the European communities, 1996, 2009; The European Commission, 2014). Pecorino Romano PDO is the most important cheese in terms of production. In fact, almost the entire amount of sheep milk produced is destined to become Pecorino Romano PDO (91%) (Laore Sardegna & ISMEA, 2016). The milk is usually inoculated with an autochthonous thermophilic culture (scotta-innesto), derived by fermentation of the residual whey from the Ricotta cheese manufacture (Pirisi et al., 2011). It is a hard semi-cooked cheese, which can be sold as a table cheese (minimum 5 months of ripening), or grating cheese (minimum 8 months of ripening), characterised by a more intense sharp taste. The main quota of Pecorino Romano PDO produced is destined for exportation. USA is the main importer of Pecorino Romano PDO, followed by France, United Kingdom, Germany, Canada and Japan (CLAL.it, 2017).

Based on the ripening period, Pecorino Sardo PDO is named “Dolce” or “Maturo”. Pecorino Sardo Dolce represents 2% of the total Sardinian PDO production, while Pecorino Sardo Mature 5%. The first one is a soft semi-cooked cheese with a maximum of 2 months of ripening (table cheese). The latter is a semi-hard semi-cooked cheese with a ripening period of longer than 2 months (table and grating cheese). Pecorino Sardo is usually produced by the inoculation

of milk with autochthonous thermophilic culture (scotta-innesto) (Pirisi et al., 2011).

Fiore Sardo PDO production represents 2% of the total Sardinian PDO production. It is produced using ancient techniques, reported in the 4th century A.D. by ancient romans. It is a hard-uncooked cheese made from raw ewe milk usually without any addition of starter culture (Pirisi et al., 2011). It can be sold as a table cheese after 3.5 months and as a grating cheese after a longer ripening period, usually from 6 months.

Table 2
Sardinian PDO cheeses (Adapted from Pirisi et al., 2011).

Cheese	Type of rennet	Type of cheese	Weight (kg)	Ripening (months)
Pecorino Romano	Lamb paste	Hard semi-cooked	20-35	5-8
Pecorino Sardo	Calf liquid	Soft semi-cooked	1-2.3	0.7-2
		Semi-hard-semi-cooked	1.7-4	> 2
Fiore Sardo	Lamb or kid paste	Hard uncooked	1.5-4	≥ 3.5

Table 3
Principal chemical composition (% w/w) of Sardinian PDO cheeses (Adapted from Pirisi et al., 2011).

Cheese	Ripening (months)	Dry matter	Protein	Fat	NaCl
Pecorino Romano	8	63.7-67.6	28.0-29.5	28-30	3.2-4.5
	12	62.2	27.2	29.7	8.7
Pecorino Sardo	2	63.1	23.8	32.1	2.1
	7	72.1	27.4	32.4	1.8
Fiore Sardo	4	65.1	25.5	33.0	2.8
	6	73.0	29.9	32.5	4.7
	9	47.9	31.1	32.8	4.9

1.7. Veterinary antibiotics

The term antibiotic is derived from ancient Greek “αντιβιοτικά”, anti “against” and bios “life”. They are molecules used to treat or to prevent bacterial infections by the inhibition of growth or killing of bacteria. In veterinary practice, they are used for the therapeutic purpose of treating pathologies or infections for a limited time. Antibiotics are also used as prophylactic agents to avoid the diffusion of diseases into the flock by the administration of low doses for a long period. Moreover, antibiotics are often employed as growth promoters by adding them to animal feed. The molecules may be used to increase the animal’s weight and this treatment can be prolonged for the entire course of the animal’s life.

In the European Union their utilization as a growth promoter has been forbidden by the application of the Reg. n. 1831/2003 (The European Parliament and the Council of the European Union, 2003), since 2006. Recently, the United States of America (U.S. Department of Health and Human Services, Administration, & Medicine, 2013) has also banned this practice, which conversely is still permitted in other countries.

It is possible to classify the veterinary antibiotics on the basis of their action mechanism, target and spectrum of action (Table 4).

Table 4
Classification of antimicrobial family utilised in veterinary medicine.

Antimicrobial class	Action	Target	Type of spectrum
Tetracyclines	Bacteriostatic	Protein Synthesis Inhibitors	Broad spectrum
Penicillins	Bactericidal	Cell Wall Synthesis	Broad spectrum
Sulfonamides	Bacteriostatic	Folic Acid synthesis inhibitors	Broad spectrum
Trimethoprim	Bacteriostatic	Folic Acid synthesis inhibitors	Broad spectrum
Macrolides	Bacteriostatic	Protein Synthesis Inhibitors	Narrow spectrum
Lincosamides	Linked to concentration applied	Protein Synthesis Inhibitors	Moderate-spectrum
Fluoroquinolones	Bactericidal	DNA Synthesis Inhibitors	Broad spectrum 3rd generation fluoroquinolones; Narrow spectrum – other fluoroquinolones
Aminoglycosides	Bactericidal (dose dependent)	Protein Synthesis Inhibitors	Broad spectrum but NOT effective against anaerobic bacteria
Polymyxins	Bactericidal	Cell Wall Synthesis	Narrow spectrum affecting primarily Gram negative bacteria
Pleuromutilins	Bacteriostatic	Protein Synthesis Inhibitors	Broad spectrum
Amphenicols	Bacteriostatic	Protein Synthesis Inhibitors	Broad spectrum
Cephalosporins	Bactericidal	Cell Wall Synthesis	Broad spectrum
Quinolones	Bactericidal	DNA Synthesis Inhibitors	Broad spectrum

1.8. Sales of veterinary antibiotics in Europe

The European Medicines Agency (EMA) monitors the sales of veterinary antibiotics in the European Union (28 countries), European Economic Area (EEA) and Switzerland. The total amount of veterinary antibiotic sales was 9009.5 tons in 2014 (latest report available) (European Medicines Agency & European Surveillance of Veterinary Antimicrobial Consumption, 2016).

As reported in table 5, Spain leads the consumption of veterinary antimicrobial agents, followed by Italy and Germany.

Table 5
Sales of veterinary antimicrobial agents in Europe for food-producing animals, express in ton of active ingredient on 2014 (EMA, 2014).

Country	Ton of veterinary	Country	Ton of veterinary antimicrobial agents
Austria	53.4	Latvia	6.3
Belgium	265.7	Lithuania	11.9
Bulgaria	32.6	Luxembourg	2.1
Croatia	40.2	Netherlands	214.5
Cyprus	41.7	Norway	5.8
Czech Republic	55.9	Poland	578.5
Denmark	106.8	Portugal	190
Estonia	9.8	Romania	98.1
Finland	11.4	Slovakia	16.3
France	761.5	Slovenia	5.7
Germany	1305.8	Spain	2936.9
Hungary	150.4	Sweden	9.3
Iceland	0.6	Switzerland	46.4
Ireland	89.6	United Kingdom	429.6
Italy	1431.6		

The amount of veterinary antibiotics sold in Europe varies depending on the country. It is possible to compare the amount of sales in relation to the number of animals by the application of the population correction unit (PCU). The European Medical Agency defines PCU as a value used "to normalise the sales by animal population in individual countries: 1 PCU equals 1 kg".

The calculation of PCU involves "multiplying numbers of livestock animals (dairy cows, sheep, sows and horses) and slaughtered animals (cattle, goat, pigs, sheep, poultry, rabbits and turkeys) by the theoretical weight at the most likely time for treatment", and total PCU is calculated as follows:

$$\text{PCU} = \text{total PCU}_{\text{domestic}} + \text{total PCU}_{\text{export}} - \text{total PCU}_{\text{import}}$$

Tetracyclines is the most sold class of antibiotic, representing 33% of the antimicrobial families administered in Europe livestock, followed by penicillins (25%), and sulphonamides (11%). These three families represent about 70% of total sales of veterinary antibiotics, as reported in figure 3 (European Medicines Agency & European Surveillance of Veterinary Antimicrobial Consumption, 2016).

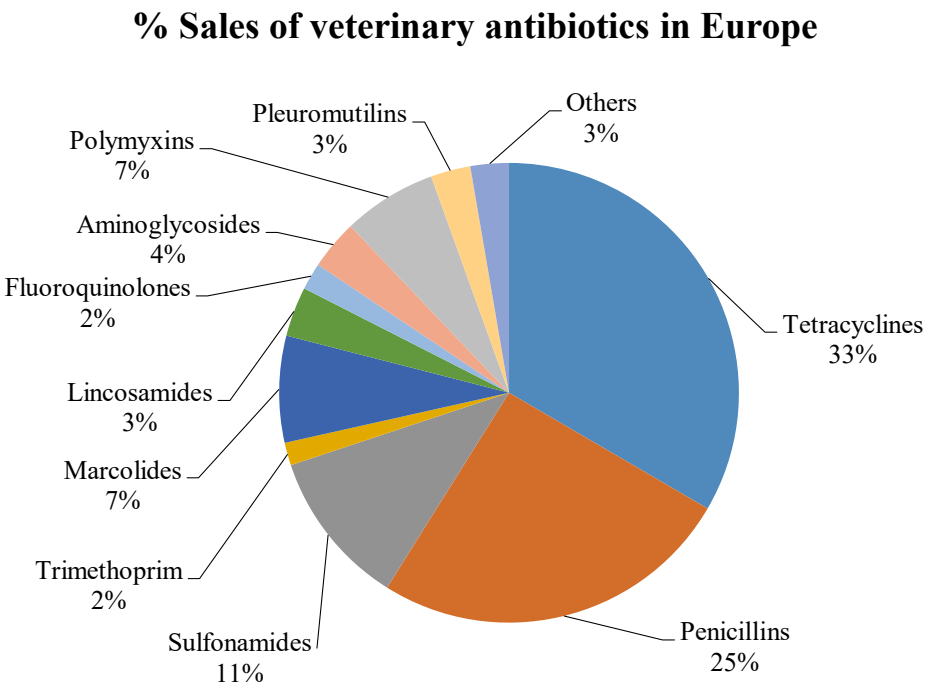

Fig. 3 Sales of antimicrobial agents divided into antimicrobial class in Europe (29 countries). Data is expressed as a percentage of total sales for food-producing animals (mg PCU^{-1}). Others represent the sum of amphenicols, cephalosporins, and quinolones (EMA, 2014).

The sales of tetracyclines constitute a great part of the veterinary antimicrobial market in Europe.

In Italy, tetracyclines represent 27.1% of total percentages of sales for food-producing animals (expressed in mg PCU^{-1}) equal to 387.7 tons of active ingredients (European Medicines Agency & European Surveillance of Veterinary Antimicrobial Consumption, 2016).

1.9. Oxytetracycline

Oxytetracycline (OTC) is the antibiotic most administered to food-producing animals, in order to treat mastitis and other infectious diseases (Naik et al., 2017).

Oxytetracycline (OTC) belongs to the tetracycline family. It is a natural polyketide, antimicrobial agent, and was discovered in 1950 produced by *Streptomyces rimosus.* Initially named Terramycin and later changed to oxytetracycline (Pickens & Tang, 2010). OTC has a broad-spectrum activity, it is used to treat many bacterial infections caused by gram-positive and gram-negative bacteria, and can also be used against atypical organisms such as, mycoplasma, rickettsiae, chlamydiae and protozoan parasites. For these reasons OTC is administrated to treat infectious animal diseases such as those of the respiratory tract, urinary tract, soft tissues, skin and mastitis in food-producing animals (Papich, 2016). Tetracyclines are administrated orally by feed, water, intramammary infusion, or via parenteral injection.

The chemical structure of oxytetracline is similar to the other tetracyclines, i.e. a linear fused tetracyclic nucleus (rings designated A, B, C, and D) to which different functional groups are attached (Fig. 4) (Chopra & Roberts, 2001). It has a molecular weight of 460.434; log P, -1.50; pKa, 7.75; log D, -4.25 (pH 7.4) (Drugbank, 2017).

OTC has a bacteriostatic action that causes the inhibition of cell growth, able to interfere with the production of essential proteins. OTC binds, in a reversible way, to the 30S ribosomal subunit and prevents the amino-acyl tRNA from binding to

the A site of the ribosome (Chopra & Roberts, 2001) by passive transport and successively active transport (Michalova, Novotna, & Schlegelova, 2004).

Oxytetracycline can easily pass through the cell membrane or passively diffuse through porin channels in the bacterial membrane (Chopra & Roberts, 2001).

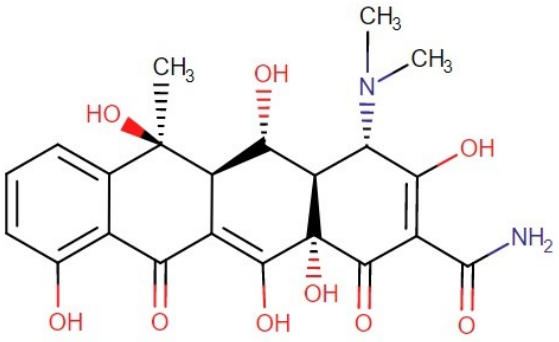

Fig. 4 Oxytetracycline structure (drugbank.com).

Tetracyclines, including oxytetracycline, are assimilated in a range from 60-90% through the upper gastrointestinal tract, and their absorption is influenced by the presence of food (Bill, 2017) or bivalent cations (Hsu, 2008). OTC is able to chelate bivalent cations such as Cu^{2+}, Fe^{2+}, Ni^{2+}, Ca^{2+} and Mg^{2+} ions (Arias et al., 2007; Comunian, Paba, Dupré, Daga, & Scintu, 2010; Martin, 1979), and this property can cause a decrease in absorption during oral administration (Papich, 2016). Tetracyclines are also able to bind animal proteins (Adetunji, 2011; Posyniak, Zmudzki, Semeniuk, Niedzielska, & Ellis, 1999).

Tetracyclines, including oxtytetracycline, are amphoteric and show variable solubility based on pH condition (Sambamurthy & Kar, 2006).

Many variables can be involved in the tetracyclines degradation, such as pH, temperature, and light. Often these factors contribute to the formation of degradation products that possess anti-microbial properties.

The epimers derived by OTC degradation, are the α-apo-OTC (α-apo-oxytetracycline), β-apo-OTC (β-apo-oxytetracycline), and 4-epi-OTC (4-epi-oxytetracycline), which have antimicrobial activity similar to the original molecule (Botsoglou & Fletouris, 2001; Halling-Sørensen, Sengeløv, & Tjørnelund, 2002). The degradation of OTC is thermal-dependent and Doi and co-workers (2000) reported in their study that a low temperature (4°C) preserves the stability of a molecule, while a higher temperature (43°C) promotes a partial degradation in aqueous solution (pH 7.0). The Doi's research group also investigated the relationship between pH and degradation rate, demonstrating that an acidic environment preserves the stability of the molecule, while an alkaline pH promotes its degradation. This behaviour was confirmed by Xuan et al (2010), who also described the role played by the presence of Ca^{2+} on degradation of OTC in the aqueous matrix (pH solutions). In fact, Xuan described the role of Ca^{2+} in causing a decrease in hydrolysis rate, and how an acidic environment protects the molecule by degradation. Zhang and his research group (2014) demonstrated the role of metal ions in OTC degradation in aqueous solution, showing that at a temperature of 40°C and 70°C, Ca^{2+}, Ni^{2+} and Zn^{2+} were able to decrease the degradation rate of OTC, while Cu^{2+} accelerated the degradation, probably through Fenton's reaction (Dzomba, Kugara, & Zaranyika, 2015).

Dzomba et al (2015) found that OTC dissolved in distilled water under natural light, underwent abiotic degradation of about 20% after 90 days.

In a recent review Heshmati et al (2015) reported various studies conducted on antibiotic drug residues in foods, including OTC. The review also reported a study conducted on foods contaminated by OTC such as bovine liver, bovine muscle and ovine muscle. The food was cooked using several different methods (boiling, grilling, microwaving, etc.), and the degradation of molecule ranged from 35% to 94%.

Moats (1999) reported some experiments performed on milk spiked with OTC (from 320 μg L^{-1} to 3220 μg L^{-1}), to evaluate the relationship between the combination of temperature and time on the elimination of the molecule. The molecule was completely eliminated after 190 min at 71°C.

1.10. Effects of the presence of antibiotic residues in milk

The wide employment of antibiotics in livestock may lead to undesirable residues in milk (Albright, Tuckey, & Woods, 1961). In fact, the presence of antibiotic residues in milk, derived from therapeutic or non-therapeutic use, can produce various negative effects on human health (antibiotic resistance, allergies, alteration of intestinal microflora), and technological problems.

Indeed, antimicrobial resistance (AMR) is a global concern because it poses a risk to human health and food safety with economic repercussions. Bacterial infections are one of the main causes of death in the world, and it is estimated that 17 million people die every year (Martens & Demain, 2017). In Europe, 25000 people die every year due to infections caused by bacteria resistant to antimicrobials. Deaths caused by resistant bacteria are equal to € 1.5 billion, calculated in medical costs and drop of production (European Medicines Agency EMA, 2017). Antimicrobial resistance in livestock represents a problem because it occurrence in zoonotic bacteria present in animals and food, which can have a negative impact on the treatment of infectious diseases in humans (European Food Safety Authority EFSA, 2017; Mąka, Maćkiw, Ścieżyńska, & Popowska, 2015). Antimicrobial resistance can reach humans through the food chain or by direct contact with colonized animals (European Medicines Agency EMA, 2017; Founou, Founou, & Essack, 2016; Liu, He, Fu, & Dionysiou, 2016). Even strains not belonging to pathogenic species (such as lactic acid bacteria), but with acquired (not intrinsic) antibiotic resistance are a risk for human health. Indeed, acquired resistances can be harboured in mobile genetic elements having a high potential for transfer of resistance to pathogenic bacteria, for example, if they come in contact with the

gastrointestinal tract (European Food Safety Authority EFSA, 2012). Moreover, residues contained in milk can produce an alteration of intestinal flora in children and in adults (Devirgiliis, Barile, & Perozzi, 2011; Rose et al., 2017).

Furthermore, most of the antibiotics are excreted from animals in active forms (Daghrir & Drogui, 2013) and residues can also be related to the contamination of the environment through urine and faeces causing adverse effects on soil microbiota (Heuer, Schmitt, & Smalla, 2011; Martínez-Carballo, González-Barreiro, Scharf, & Gans, 2007; Ventola, 2015; Zhang et al., 2014)

In order to guarantee human health and minimize the presence of antibiotic residues in milk, their concentration must not exceed the Maximum Residue Limits (MRLs) fixed by the European Union for veterinary medical products in foodstuffs of animal origin (The European Commission, 2010). The MRL is the maximum concentration of pharmacologically active substance permitted in the animal matrix, expressed as $\mu g\ kg^{-1}$, and considered safe for the health of the consumer. The MRL values are based on the acceptable daily intake (ADI) of food containing an amount of residue, which if consumed for a long period does not represent a hazard to human health (Cerniglia & Kotarski, 2005).

ADI values are estimated on the basis of a toxicological evaluation of many parameters regarding drugs, dosage, administrations and animals, in order to protect human health (FAO/WHO Joint Expert Committee on Food Additives JECFA, 2002). ADI values are expressed as residue concentration ($\mu g\ kg^{-1}$) per kg of body weight, ingested daily over a lifetime without significant health risk.

ADI is calculated as follows:

$$Lower\ limit\ of\ ADI = 0$$

$$Upper\ limit\ of\ ADI = \frac{MIC_{50}\ x\ MCC}{FA\ x\ SF\ x\ BW}$$

MIC_{50} = minimum inhibitory concentration for 50% of strains of the most sensitive relevant organism;

MCC = mass of colonic contents;

FA= available fraction of the dose;

SF= safety factor, the magnitude of value depends on the quality and quantity of the microbiological data available;

BW= body weight estimated of 60 kg for an adult.

In addition to the health aspects, the presence of antibiotics in milk can produce adverse technological effects. In fact, residues can generate an inhibition or slowing down effect on the development of starter cultures, during the acidification phase of fermented dairy products such as butter, cheese and yogurt. However, a potential negative technological effect caused by the presence of residues, depends on the concentration and class of antibiotics. In fact, Berruga et al. (2007) reported in a study developed on sheep's milk yogurt, a delay during the acidification process caused by ampicillin and penicillin G, but no effect was noted with amoxicillin. Novés et al. (2015), assessed the effect of cephalexin in yogurt made from ewe milk, and observed an inhibition of *Streptococcus thermophilus* normal growth with modification in acidity parameters and texture in the final product. The action of residues can also produce a delay during acid

coagulation or pH lowering in pressed cheese, affecting the sensory properties of cheeses (Berruga, Molina, Althaus, & Molina, 2016; Nagel et al., 2009). In fact, irregular acidification can cause an early blowing defect in cheese during the first hours or first days of ripening. Early blowing defects are generally caused by coliform bacteria and yeasts, with a production of CO_2 and in particular H_2.

Despite the large amount of milk which is subjected to heat treatment in the dairy industry, the temperatures applied are not sufficient to eliminate all antibiotic residues content in milk (Hassani, Lázaro, Pérez, Condón, & Pagán, 2008; Kellnerová, Navrátilová, & Borkovcová, 2015; Shahani, 1958).

1.11. Legislative aspects

Milk is a food with excellent nutritional properties but it is exposed to a large source of risks that may compromise its quality, such as microorganisms, residues of antibiotics, pesticides, toxins, detergents, etc.

In order to guarantee the public health and the high quality of the foodstuffs for human consumption, the European Union legislated by Regulation (EC) n. 852/2004 (The European Parliament and the Council of the European Union, 2004a), Regulation (EC) n. 853/2004 (The European Parliament and the Council of the European Union, 2004b), Regulation (EC) n. 854/2004 and Regulation (EC) n. 882/2004. The Regulation (EC) n. 854/2004 and the Regulation (EC) n. 882/2004 have both recently been substituted by Regulation (EC) n. 2017/625 (The European Parliament and the Council of the European Union, 2017).

The European Union, in order to ensure food safety, has laid down Community procedures for the establishment of residue limits of pharmacologically by Regulation (EC) n. 470/2009 (The European Parliament and the Council of the European Union, 2009).

The Commission Regulation (EU) n. 37/2010 (The European Commission, 2010), lists the pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. Commission Regulation (EU) n. 37/2010 classifies the residues into two groups: allowed and prohibited substances. The prohibited substances do not have a MRL because their use is not permitted, at all. Regarding the allowed pharmacologically active substances, the

regulation classifies the molecule based on marker residue, animal species, MRL (μg kg^{-1}), target tissues, other provisions, and therapeutic classification.

For milk of all food-producing species, the MRL of oxytetracycline is fixed in milk of all food-producing species at 100 μg kg^{-1} as sum of parent drug and its 4-epimer by the Regulation n. 37/2010. The OTC is classified as an anti-infectious agent and antibiotic, and the ADI is fixed in a range of 0-30 μg kg^{-1} of body weight.

The MRL of oxytetracycline is also fixed at 100 μg kg^{-1} in Australia, Japan, China, Switzerland and Mercosur area. In USA, the MRL of OTC in milk is set at 300 μg kg^{-1}, while in the Russian Federation the presence of OTC in milk is not permitted. Unfortunately, in other parts of world the presence of antibiotic residues in milk represent a great issue from sanitary, technological and economical aspect. This problem is caused by lack of monitoring, legislation and professional formation of the operators of sector (Abbasi, Babaei, Ansarin, Nourdadgar, & Nemati, 2011; Mensah et al., 2014).

In Italy, the "Piano Nazionale per la ricerca dei residui" (PNR) has been published every year, since 1988, in order to guarantee the public health and safety of foodstuffs of animal origins by monitoring the presence of contaminants in food (Fig. 5). The program pays attention to residues of prohibited substances, abusive administration of authorized substances, residues of veterinary drugs conforming to the MRL, and the presence of environmental contaminants. PNR is coordinated by the Italian Health Ministry in collaboration with local health divisions (Assessorati Regionali alla Sanità), local survelliances (Nuclei Operativi

Regionali di Vigilanza, NORV), health veterinary departments (Servizi veterinary) and Istituti Zooprofilattivi Sperimentali.

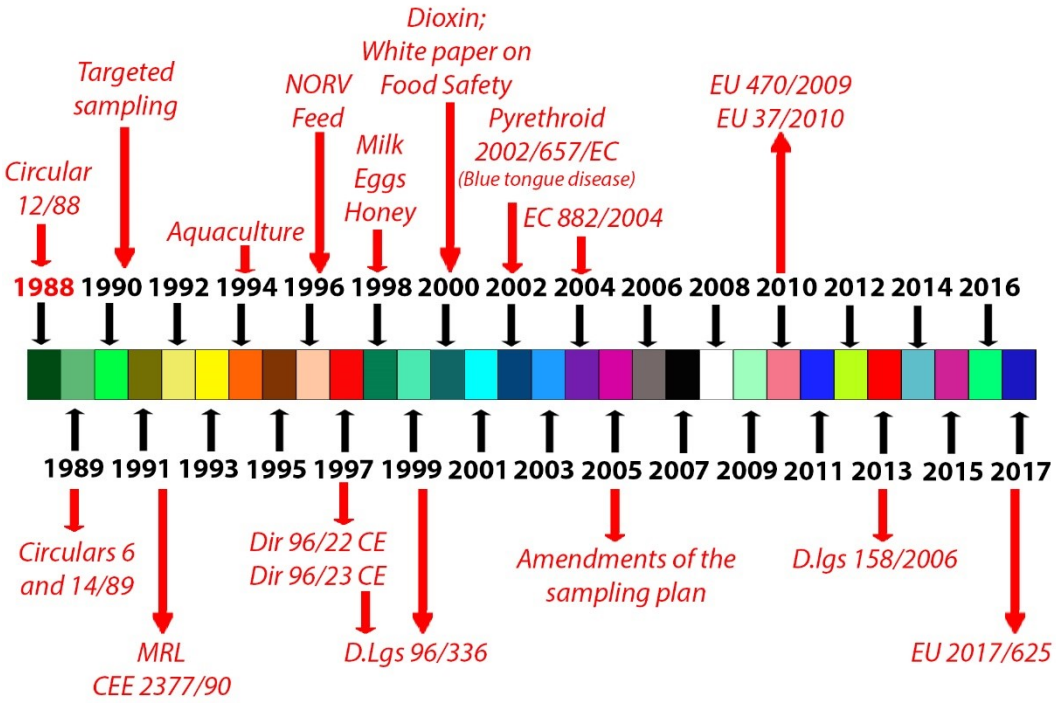

Fig. 5 Timeline of Italian "Piano nazionale dei residui PNR" from 1988 to 2017.

In addition, the "Conferenza permanente per i rapporti tra lo Stato, le Regioni e le Province Autonome di Trento e Bolzano (La conferenza permanente per i rapporti tra lo Stato, le Regioni e le Province Autonome di Trento e Bolzano, 2013)" constitutes an agreement between the Italian government and the regional administrations. In this agreement, the guidelines to improve official controls by the competent authorities regarding both the public veterinary health and food safety are reported. In 2014, Sardinian region (Regione Autonoma della Sardegna, 2014) approved the new guidelines in order to regulate the controls and guarantee

the food safety of milk destined to undergo thermal treatments and transformation. The guidelines state a system of self-checking, with a sampling rules that all the parties involved (farmers, dairy cheese-making factories and milk collection centres, associations) must comply. Regarding veterinary residues the adopted system allow a continuous capillary control of the milk of each farm ensuring an early alert in case of positive samples.

2 Aim of the project

Veterinary antibiotics are an important tool used to treat infectious diseases, prophylactic agents, and feed additives to promote growth in livestock. However, they should be administrated with caution, in order to limit their presence in products of animal origins. The antibiotic residues in milk represent a risk to the public health. In fact, food contaminated by antibiotics, even at low levels, may increase the risk of antimicrobial resistance (AMR). Actually, AMR is a global threat to the human health. Consummation of contaminated food can cause acute allergic reactions in sensitive consumers. To control the problem, the European legislation (UE 37/2010) regulates the maximum residue limit (MRL) for the pharmacologically active substances in foodstuffs of animal origins (including veterinary antibiotics) such as milk, honey, meat and other edible tissues. Although MRLs are expressed for foodstuffs of animal origin, for instance the milk of many species, to date no MRLs are listed by the European Union for derivative food, including dairy products, such as cheese, whey, yogurt, butter and others. This legislative void complicates the decision-making on residues by the official control entities, as well as the international trade agreements.

Antibiotic residues present in milk, even close to the MRL, can produce negative effects during the transformation of milk. These effects can cause interference with the growth of bacteria during the acidification process and therefore lead to defects in the final product.

The available literature about the presence and the transfer of drugs residues from milk to milk-derivatives, and their partition between the obtained fractions of the process (whey, curd, etc.), is scarce. To date, only a few studies have focused on the

relationships between low concentrations of antibiotic residues in milk and their adverse technological effects on dairy processing.

The tetracycline family represents the most sold and prescribed antimicrobials in livestock both in Italy and Europe.

In Sardinia, the oxytetracycline (OTC) is one of the most used antibiotics in ovine and caprine livestock (42%). This data has been elaborated from a preliminary investigation about veterinary prescriptions in 2016.

In this context, the overall objective of this Ph.D. thesis was to study the distribution of oxytetracycline, previously added to ovine milk at legal concentrations (MRL level or below), among the obtained fractions of the cheese-making process. As for the reasons explained in section 1.11, the MRL level of OTC contamination in milk chosen in this study is difficult to reach in industrial production (worst-case scenario), while easily achievable in artisanal production. The effects on both cheese composition and microflora were evaluated during cheese-making and in the ripened cheese. In order to accomplish the overall purpose, the research project was divided into four different parts, each of which with a specific aim.

In the first part of the project a preliminary study was conducted in order to evaluate the distribution of OTC spiked at MRL level, between whey and curd in laboratory scale cheese-making.

In the second part of the project experimental cheese-makings were performed, in a pilot plant, starting from milk spiked with OTC at MRL level. The experiments were conducted by a conventional cheese-making process based on raw milk used to produce

an uncooked cheese. The experiments were conducted with milk collected from an untreated flock of Sarda breed sheep. The raw milk was divided into two aliquots, the first one unspiked and the second one with addition of OTC, which underwent the cheese-making process in order to obtain an uncooked hard cheese from ovine raw milk. The OTC was measured in all the obtained fractions and in the ripened cheese, and then the overall distribution of the molecule was calculated. Furthermore, the effect throughout the cheese-making process and on cheese composition was assessed and the potential implications for human health were discussed. It was the first study on the fate of antibiotics in ovine cheese, conducted in real cheese-making conditions.

In the third part of the study, the ability of the thermal treatment (thermisation) to reduce the content of OTC residues in milk and its impact on the distribution of the molecule throughout the cheese-making and in ripened cheeses were investigated. In literature most of the studies focus on the effect of thermal treatments such as pasteurization and UHT and their correlation with the degradation rate of OTC, which is present in cow milk. In addition, thermisation of ovine milk is a common practice in Sardinia. It was applied the cheese-making adopted in the previously experiment, with the introduction of a thermal treatment of milk, in order to obtain an uncooked cheese.

The initial spike on raw ovine milk was performed at two levels, MRL and half MRL. After the milk's thermisation, the fate of residues into the obtained fractions of the process and ripened cheeses were monitored. Moreover, the effects of the OTC residues on microbiota of cheeses were studied by viable plate counts.

In the fourth part of the study, the effects caused by the presence of OTC at MRL level, during the early phase of acidification and on the development of the thermophilic starter culture were investigated.

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4 Chapter I: Transfer of veterinary antibiotics from ovine milk to curd. A preliminary investigation

1. Introduction

Antibiotics are widely used in dairy small ruminant livestock to treat mastitis and other infectious diseases, but their administration should be carefully managed in order to limit the occurrence of their residues on products of animal origin.

The presence of veterinary antibiotics in milk and milk derivatives represent a risk for human health, since it may lead to bacteria resistance phenomena and allergic reactions in highly sensitive consumers (Adetunji, 2011). The Commission Regulation (EU) 37/2010 (The European Commission, 2010) states the Maximum Residue Limit (MRL) on pharmacologically active substances in foodstuffs of animal origin, included milk, but to date no limits are specified for milk derivatives such as cheese. This regulatory gap may complicate both the decision-making on residues by the official control entities, as well as the international trade agreements.

Additionally, the antibiotic residues in milk, even at MRL level, can have negative technological implications during the manufacturing of dairy products, as showed by Berruga et al. (2016).

The available literature about the technological effects of antibiotics on cheese obtained from milk close to MRL level is scarce. Oxytetracycline is a bacteriostatic agent characterised by effectiveness, low cost and user-friendly formulations. The Commission Regulation (EU) 37/2010 set the MRL of OTC in milk at 100 μg kg^{-1}, expressed as the sum of parent drug oxytetracycline and its epimers, 4-epi-oxytetracycline (EOTC), α-apo oxytetracycline (α-apo-OTC) and β-apo-oxytetracycline (β-apo-OTC).

OTC is one of the most used antibiotics in Sardinia ovine caprine livestock, and for this reason it was selected in this preliminary study conducted in sheep milk. Laboratory cheese-making method (Manca et al., 2016), was used as a tool for a preliminary investigation in order to understand the partition of the molecule between whey and cheese.

2. Materials and Methods

2.1 Chemical and reagents

Oxytetracycline hydrochloride (OTC, purity ≥96.7%) was purchased from Sigma-Aldrich (St. Louis, MO, USA); 4-epi-oxytetracycline (EOTC, purity ≥97%), α-apo-oxytetracycline (a-apo-OTC) and β-apo-oxytetracycline (b-apo-OTC) were purchased from Acros Organics (Geel, Belgium). All solvents used were high performance liquid chromatography (HPLC) grade from Carlo Erba (Milan, Italy), and liquid chromatography-mass spectrometry (LC-MS) grade water was produced with an Advantage System (Millipore, Billerica, MA, USA). Stock solutions of OTC, α-apo-OTC and β-apo-OTC, EOTC were prepared by diluting the pure standard in methanol to a final concentration of 1 g L^{-1} and stored in the dark at -20 °C until their use for the analytical procedure. Immediately before each cheesemaking trial a spiking-solution was prepared by diluting the stock solution with LC-MS grade water to a final concentration of 0.12 g L^{-1}.

2.2 Lab-scale cheese-making

Ovine antibiotic-free milk was collected from a flock of healthy and untreated ewes at the experimental farm of AGRIS Sardegna Research Agency (Olmedo, Sassari, Italy). The mean chemical composition of the milk was as follows: pH 6.67 ± 0.10; total solids, 15.78 ± 0.07% (w/w); protein 4.96 ± 0.02% (w/w); fat, 5.44 ± 0.03% (w/w); casein, 3.80 ± 0.04% (w/w); and lactose 4.66 ± 0.03% (w/w). Fresh whole raw ovine milk was spiked with OTC at MRL level (100 µg kg^{-1}) and divided into 12 aliquots of 10 g each. Aliquots were coagulated simultaneously in a water bath, at 36°C by addition of 0.600 IMCU ml^{-1} of liquid rennet (CHY-MAX M1000, CHR HANSEN, Denmark). Milk was converted into curd using the process described in figure 6 (Manca et al., 2016). Lab-scale cheesemakings were replicated 3 times.

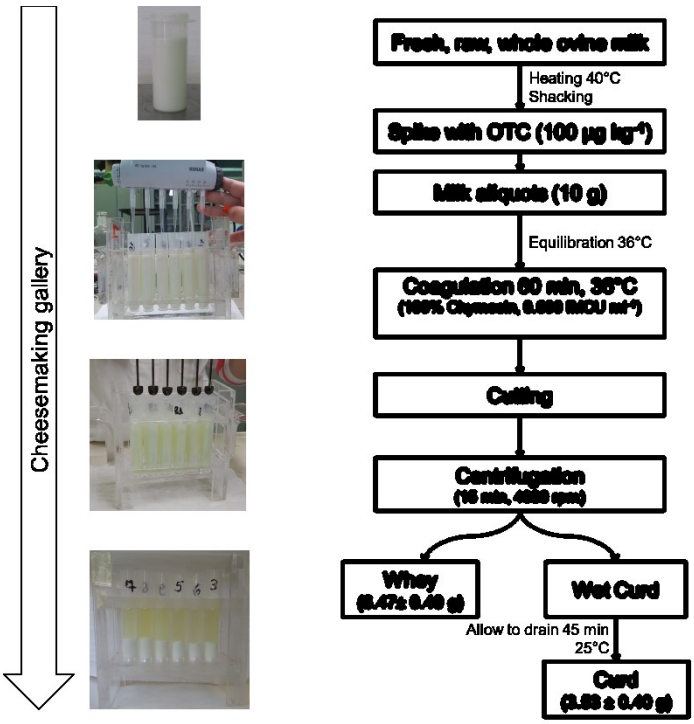

Fig. 6 Lab-scale cheese-making.

2.3 Chemical Analysis

The levels of fat, protein and casein in milk were measured using the MilkoScan FT+ (Foss, Hillerød, Denmark) according to the method ISO 9622 of the International Organisation for Standardisation (ISO, 2013). Total solids were measured by gravimetric analysis after drying to constant weight at 102 ± 2 °C according to the ISO method 6731:2010 (ISO, 2010). pH was measured using pHmeter (Crison Basic 20+, equipped with a T electrode, Crison Instruments, Barcelona, Spain). Samples of whey and curd were tested for: dry matter according to ISO 5534 (ISO, 2004); fat (Soxhlet, 1879), protein according to ISO 8968-1 (ISO, 2014). Each analysis was performed in duplicate.

2.4 Measurement of OTC levels in milk, whey and cheese

2.4.1 Sample extraction and solid phase extraction parameters

Milk and whey samples (5.0 ± 0.1 g) were weighed into a 25 mL centrifuge tube; 8.3 mL of Mc Ilvaine EDTA buffer (pH 4) were then added and mixture was agitated using a vortex (Classic Advanced Vortex Mixer, Velp Scientifica, Usmate Velate, MB) and centrifuged at 10000 rpm for 10 min at 0 °C (Centrifuge 5810R, Eppendorf, Hamburg, Germany). The supernatant was purified using a Gilson automatic sample processor ASPEC on 3 mL OASIS HLB SPE cartridges, previously conditioned with 3 mL MeOH and 2 mL H_2O. The sample (5 mL) was then loaded on the cartridge, subsequently washed with 1.5 mL H_2O:MeOH (95:5, v/v). The analytes were eluted with 2 mL MeOH into conical-bottom centrifuge tubes. The eluates were then evaporated near to dryness under a gentle stream of nitrogen, reconstituted into 1 mL

14% MeOH (in water), and filtered on a 0.20 mm Hydrophilic PTFE Millex Samplicity filter (Millipore).

Curd homogenized samples (1.0 ± 0.1 g) were weighed into a 7 ml Precellys tube (Bertin Technologies, Aix-en-Provence, France), extracting with 5 mL of Mc Ilvaine EDTA buffer (pH 4) at 5000 rpm for 20 s at 0°C for two times, and then centrifuged at 10000 rpm for 10 min at 0°C. Supernatant was transferred into a glass centrifuge tube. The extraction process was repeated and the combined extracts were purified, as described above for milk and whey samples.

2.4.2 Liquid chromatography-tandem mass spectrometry analysis

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed using an Acquity UPLC I Class system coupled to a Micromass Quattro Premier XE Triple Quadrupole mass spectrometer (Waters, Milford, MA, USA). The LC configuration was as follows: Waters Acquity BEH C18 (100 mm x 2.1 mm, 1.7 µm particles) column preceded by a guard column BEH Shield RP18 (1.7 mm 2.1 mm, 5 µm particles), gradient elution with H_2O with 0.1% (v/v) formic acid (A) and MeOH (B). The injection volume was 10 µL, the flow rate was 0.450 mL min^{-1}, and the adopted gradient varied the % of A to B as follows: T = 0.0 min, 95%; T = 1.94 min, 95%; T = 6.19 min, 25%; T = 7.32 min, 25%; T = 7.38 min, 0.0%; T = 7.89 min, 95%; T = 10.00 min, 95%, the target column temperature was 40 C.

The electrospray ionisation (ESI) source was operated in positive ion mode, with the following MS conditions: ion-source temperature, 120 °C; desolvation temperature, 350 °C; desolvation gas flow, 900 L h^{-1}; cone gas flow, 50 L h^{-1}; collision gas flow, 0.21 mL

min^{-1}. Multiple reaction monitoring (MRM) was used and two transitions per analyte were selected for identification (OTC and EOTC 461.00 > 426.00, 461.00 > 443.00; α-apo-OTC and β-apo-OTC 443.40 > 426.10, 443.40 > 229.00), but only the most intense transition was used for quantification. The cone was kept at 25 V and the most suitable collision energy was selected in the range 13-20 eV.

2.4.3 Method validation

An in-house validation protocol was carried out to establish the performance characteristics of the method, ensuring adequate identification, confirmation and quantification of OTC. The method was validated in milk, whey and cheese by the criteria described below. The selectivity and specificity were assessed by analysing 6 blank samples from each matrix. The absence of background peaks, above a signal-to-noise ratio of 3, at the retention time of OTC showed that the method was free of endogenous interferences. The trueness was determined by analysing 6 separate samples of milk, whey and curd that were each spiked with OTC at a level of 100 µg kg^{-1}, equivalent to the MRL of OTC in milk. The obtained trueness, expressed as the percent of recovery was 85, 90 and 60 for milk, whey and curd, respectively. The limit of quantification (LOQ) was defined as the lowest concentration or mass of the analyte that has been validated with acceptable accuracy, by applying the complete analytical method. The calculated LOQ was 10 µg kg^{-1} for milk and whey, and 40 µg kg^{-1} for curd. The repeatability, expressed as the relative standard deviation "within-lab" repeatability (RSDwr; %), was 14% for milk and whey and 16% for cheese.

2.5 OTC mass balance

Molecule mass balance was expressed as a percentage ratio (w/w) between recovery of molecule and the output products: whey, curd and total processed milk weight.

3. Results

Lab-scale cheese-making was characterized by a good repeatability (< 4%) calculated as the variation coefficient of the yield of the cheese-making, which was simultaneously performed on 12 test tubes, as reported by Manca et al. (2016). Chemical compositions of whey and curd are reported in table 6. The average cheese yield (36.6 ± 3.6%) was higher than that obtained in real cheese-making conditions. This result was influenced by a small volume of milk. In addition, in laboratory conditions draining is accelerated and enhanced by centrifugation, while in real cheese-making this phase can last some hours, depending on type of cheese (Othmane, Carriedo, De la Fuente Crespo, & San Primitivo, 2002).

Additionally, in real cheese making there also other stages present, such as breaking of the clot and consequently syneresis, acidification phase, post-sweating stage, pressing and salting phases.

Table 6
Chemical compositions (%, w/w) of fraction obtained from lab-scale cheese-making.

Parameter	Whey	Curd
Dry matter	6.73 ± 1.08	33.41 ± 1.43
Fat	0.41 ± 0.07	14.64 ± 2.17
Protein (NT x 6.38)	1.75 ± 0.31	11.85 ± 0.80

Data obtained in the above described conditions, showed that about the 37% (105.1 ± 8.4 μg kg^{-1}) of the total amount of the total added OTC (1 μg) was detected in the curd, while the 18% (27.9 ± 0.6 μg kg^{-1}) was recovered in the whey. A significantly high quote of the total added OTC (about 45%) was not recovered. This may be due both to the limits of lab-scale volumes and to the uncertainty of the analytical method. No epimers were found in fractions. These results support the hypothesis that the molecule has great affinity with milk useful matter. Oxytetracycline has characteristics to bind protein animals and bivalent cations, present in high concentrations in cheese.

4. Conclusions

Lab-scale cheese-making was a preliminary screening tool useful to understand the possible fate of OTC added in milk. A significant quote of total OTC spiked in milk was recovered in curd (37%), but a high quote was not recovered. Further studies will be needed to better understand the fate of the molecule in real cheese-making conditions.

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5 Chapter II: Transfer of oxytetracycline from ovine spiked milk to whey and cheese

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Transfer of oxytetracycline from ovine spiked milk to whey and cheese

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ABSTRACT

Cheese-making trials with ovine milk spiked with oxytetracycline (OTC) at maximum residue limit (MRL) level were performed to study the OTC distribution between whey and cheese, and assess its effect in cheese-making process and cheese composition. An in-house validated method, based on liquid chromatography-tandem mass spectrometry, was adopted to detect OTC in milk, whey and cheeses. The MRL OTC spike induced a delay in the pH lowering causing a higher moisture content in 1-day OTC cheese compared with the control cheese. No effects of OTC were observed on the mass balance of the process, on the recovery of fat and protein and, in general, on physico-chemical parameters and gross composition of obtained cheeses. OTC was mainly recovered in the 1-day cheeses (61 ± 5%). Despite a 17 ± 8% OTC reduction observed during cheese ripening, probably due to partial degradation, remaining residues were not negligible and could contribute to reach the acceptable daily intake.

1. Introduction

Antibiotics are widely used in dairy small ruminant livestock to treat mastitis and other infectious diseases, but their administration should be carefully managed to limit the occurrence of their residues on products of animal origin (Fletouris, Papapanagiotou, & Nakos, 2008; Molina, Molina, Althaus, & Gallego, 2003).

The presence of antibiotics residues in milk may pose a serious threat to public health. Food contaminated with antibiotics, even at low levels, could be a reservoir of antibiotic-resistant bacteria and, therefore, be one of the causes of the development of antimicrobial resistance (AMR), which is nowadays of major concern in humans, worldwide (Berruga, Molina, Althaus, & Molina, 2016; Marshall & Levy, 2011; WHO, 2014). Furthermore, the consumption of antibiotic-contaminated milk-derived foods can increase the risk of acute allergic diseases in sensitive consumers (Khosrokhavar et al., 2008).

The Annex to Commission Regulation (EU) No. 37/2010 (European Commission, 2010) lists the maximum residue limit (MRL) for pharmacologically active substances in foodstuffs of animal origin, including milk. However, currently, no limits are indicated for milk-derived foods, and this regulatory gap may complicate both the decision-making on residues by the official control entities, as well as the international trade agreements. Antibiotics residues, even close to the MRL, may have a negative impact on the processing of milk by interfering with the growth of bacteria, for example during cheesemaking (Berruga, Battacone, Molina, Román, & Molina, 2008). Their action may result in delayed acid coagulation or delayed pH lowering in pressed cheese, as recently reviewed by Berruga et al. (2016) on sheep and goat milk.

Oxytetracycline (OTC) is a broad-spectrum antibiotic belonging to the tetracycline family, which acts as bacteriostatic, since it inhibits cell growth by interfering with the ability of bacteria to produce essential proteins. It binds to the 30S ribosomal subunit and prevents the amino-acyl tRNA from binding to the A site of the ribosome (Chopra & Roberts, 2001). In many countries OTC and other antibiotics are used as a growth promoter, added at sub-therapeutic levels to animal feeds, while this use is forbidden in Europe since 2006 (EPCEU, 2003). The Commission Regulation 37/2010 (European Commission, 2010) and Codex Alimentarius (2015) set the MRL of OTC in milk at 100 μg kg^{-1}, expressed as the sum of parent drug oxytetracycline and its epimers, 4-epi-oxytetracycline (EOTC), α-apo-oxytetracycline (α-AOTC) and β-apo-oxytetracycline

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(β-AOTC), while the USA Food and Drug Administration (FDA, 2012) sets a limit of 300 μg kg^{-1}. OTC, due to its effectiveness, low cost and the user-friendly available formulations, is often used to treat dairy diseases in sheep, such as mastitis and gastrointestinal, respiratory, genitourinary and systemic infections; it is one of the most prescribed veterinary antibiotics in Sardinia.

The available literature about the transfer of drugs residues from milk to milk-derivatives (e.g., cheese, yogurts, etc.), and their partition among the obtained fractions (e.g., whey, curd), is scarce and mainly focused on antiparasitics (Hakk et al., 2016). To date, only a few studies have focused on the relationships between low concentrations of antibiotics residues in milk and their adverse technological effects on dairy processing (Berruga et al., 2016).

The island of Sardinia is the Italian region with the largest quantity of ewe milk collected annually, equivalent to about 70% of national production; this milk is destined almost exclusively for cheese-making (Pirisi, Comunian, Urgeghe, & Scintu, 2011).

The aim of the current study was to study the distribution of oxytetracycline (OTC), added to ovine milk at a concentration near to the MRL, between whey and cheese, and to establish its effect on the cheese-making process and on cheese composition.

2. Material and methods

2.1. Chemicals and reagents

Oxytetracycline hydrochloride (OTC, purity ≥96.7%) was purchased from Sigma–Aldrich (St. Louis, MO, USA); 4-epi-oxytetracycline (EOTC, purity ≥97%), α-apo-oxytetracycline (α-apo-OTC) and β-apo-oxytetracycline (β-apo-OTC) were purchased from Acros Organics (Geel, Belgium). All solvents used were high performance liquid chromatography (HPLC) grade from Carlo Erba (Milan, Italy), and liquid chromatography-mass spectrometry (LC-MS) grade water was produced with an Advantage System (Millipore, Billerica, MA, USA). Stock solutions of OTC, α-apo-OTC and β-apo-OTC, EOTC were prepared by diluting the pure standard in methanol to a final concentration of 1 g L^{-1} and stored in the dark at −20 °C until their use for the analytical procedure. Immediately before each cheese-making trial a spiking-solution was prepared by diluting the stock solution with LC-MS grade water to a final concentration of 0.12 g L^{-1}.

2.2. Cheese manufacture

Antibiotic-free ewes' milk, collected at the experimental farm of AGRIS Sardegna Research Agency (Olmedo, Sassari, Italy), was from a flock of healthy and untreated ewes. The mean chemical composition of the milk was as follows: pH 6.69 ± 0.01; total solids, 15.5 ± 0.5% (w/w); protein, 5.2 ± 0.2% (w/w); fat, 5.3 ± 0.5% (w/w); casein, 3.9 ± 0.2% (w/w); and lactose, 4.5 ± 0.3% (w/w). Bulk raw milk was divided into 2 portions of 12 kg each, one of which was a control (not spiked) and the other (treated) was spiked with oxytetracycline (MRL OTC) at the MRL level (100 μg kg^{-1}) by adding 10 mL of the previously-described spiking-solution. Both portions were converted into cheese (two loafs of about 0.9 kg from each one) using the make-procedure described in Fig. 1; cheesemaking trials from both the control and antibiotic-spiked milk samples were undertaken on 6 different occasions. Samples of cheese and whey were immediately stored at −20 °C until required for analysis.

2.3. Milk and cheese composition

The levels of fat, protein and casein in milk were measured using the MilkoScan FT+ (Foss, Hillerød, Denmark) according to the method ISO 9622 of the International Organisation for Standardisation (ISO, 2013). Total solids were measured by gravimetric analysis after drying to constant weight at 102 ± 2 °C according to the ISO method 6731:2010 (ISO, 2010). pH was measured using pH-meter (Crison Basic 20+, equipped with a T electrode, Crison Instruments, Barcelona, Spain). Samples of each cheese at 1 and 90 days were tested for: pH (pHmeter Crison Basic 20+, equipped with a 5232 Puncture electrode, Crison Instruments, Barcelona, Spain); dry matter according to ISO 5534 (ISO, 2004); fat (Soxhlet, 1879), protein according to ISO 8968-1 (ISO, 2014); pH 4.6-soluble N, 12% trichloroacetic acid soluble N, 5% phosphotungstic acid-soluble N as described by Gripon, Desmazeaud, Le Bars, and Bergere (1975); total ash by gravimetric analysis after ashing the sample at 550 °C for 12 h according to ISO 27:1964 (ISO, 1964); and NaCl according to ISO 5943:2006 (ISO, 2006). Each analysis was performed in duplicate.

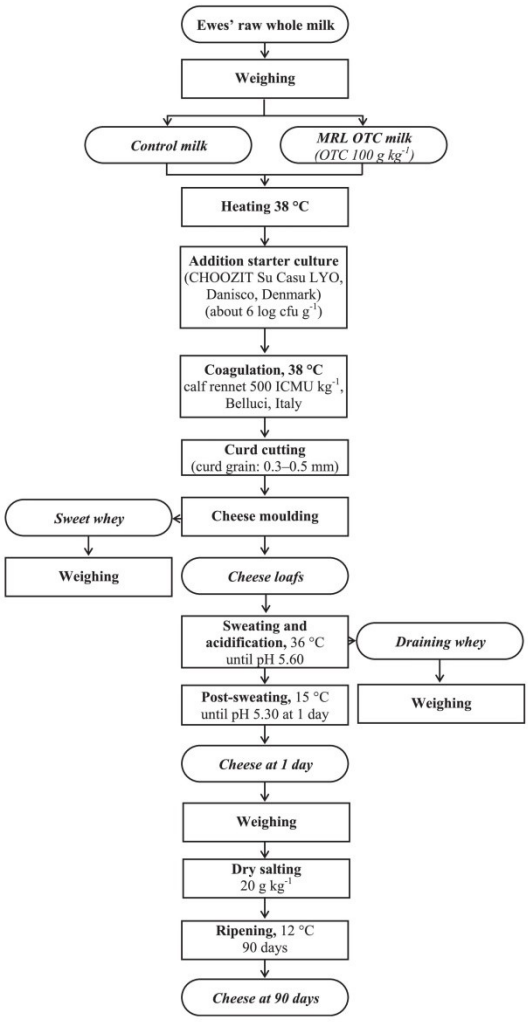

Fig. 1. Cheese making process.

2.4. Mass balance and cheese component recoveries (fat and protein)

Mass balance was expressed as a percentage ratio (w/w) between output products: sweet whey (whey collected after the forming step), draining whey (whey drained from the cheese over the first 24 h), cheese, and total processed milk weight.

The component recoveries (fat and protein) in 1-day-old cheese were estimated, as described in Guinee, Mulholland, Kelly, and Callaghan (2007).

2.5. Measurement of OTC levels in milk, whey and cheese

2.5.1. Sample extraction and solid phase extraction parameters

Milk and whey samples (5.0 ± 0.1 g) were weighed into a 25 mL centrifuge tube; 8.3 mL of Mc Ilvaine EDTA buffer (pH 4) were then added and mixture was agitated using a vortex (Classic Advanced Vortex Mixer, Velp Scientifica, Usmate Velate, MB) and centrifuged at 12,800 × *g* for 10 min at 0 °C (Centrifuge 5810R, Eppendorf, Hamburg, Germany). The supernatant was purified using a Gilson automatic sample processor ASPEC on 3 mL OASIS HLB SPE cartridges, previously conditioned with 3 mL MeOH and 2 mL H_2O. The sample (5 mL) was then loaded on the cartridge, subsequently washed with 1.5 mL H_2O:MeOH (95:5, v/v). The analytes were eluted with 2 mL MeOH into conical-bottom centrifuge tubes. The eluates were then evaporated near to dryness under a gentle stream of nitrogen, reconstituted into 1 mL 14% MeOH (in water), and filtered on a 0.20 μm Hydrophilic PTFE Millex Samplicity filter (Millipore).

The extraction of OTC from the cheese involved weighing a sample of the grated cheese (1.0 ± 0.1 g) into a 7 mL Precellys tube (Bertin Technologies, Aix-en-Provence, France), extracting with 5 mL of Mc Ilvaine EDTA buffer (pH 4) at 2000 × *g* for 20 s using a Precellys 24 System homogenizer (Bertin Technologies), centrifuging the resultant homogenate at 12,800 × *g* for 10 min at 0 °C, and transferring the supernatant into a glass centrifuge tube. The extraction process was repeated and the combined extracts were purified, as described above for milk and whey samples.

2.5.2. Liquid chromatography-tandem mass spectrometry analysis

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed using an Acquity UPLC I Class system coupled to a Micromass Quattro Premier XE Triple Quadrupole mass spectrometer (Waters, Milford, MA, USA). The LC configuration was as follows: Waters Acquity BEH C18 (100 mm × 2.1 mm, 1.7 μm particles) column preceded by a guard column BEH Shield RP18 (1.7 mm × 2.1 mm, 5 μm particles), gradient elution with H_2O with 0.1% (v/v) formic acid (A) and MeOH (B). The injection volume was 10 μL, the flow rate was 0.450 mL min^{-1}, and the adopted gradient varied the % of A to B as follows: T = 0.0 min, 95%; T = 1.94 min, 95%; T = 6.19 min, 25%; T = 7.32 min, 25%; T = 7.38 min, 0.0%; T = 7.89 min, 95%; T = 10.00 min, 95%, the target column temperature was 40 °C.

The electrospray ionisation (ESI) source was operated in positive ion mode, with the following MS conditions: ion-source temperature, 120 °C; desolvation temperature, 400 °C; desolvation gas flow, 900 L h^{-1}; cone gas flow, 50 L h^{-1}; collision gas flow, 0.21 mL min^{-1}. Multiple reaction monitoring (MRM) was used and two transitions per analyte were selected for identification (oxytetracycline and EOTC 461.00 > 426.00, 461.00 > 443.00, α-apo-OTC and β-apo-OTC 443.40 > 426.10, 443.40 > 229.00), but only the most intense transition was used for quantification. The cone was kept at 25 V and the most suitable collision energy was selected in the range 13.0–36.0 eV.

2.5.3. Method validation

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

The selectivity and specificity were assessed by analysing 6 blank samples from each matrix. The absence of background peaks, above a signal-to-noise ratio of 3, at the retention time of OTC showed that the method was free of endogenous interferences. The trueness was determined by analysing 6 separate samples of milk, whey and cheese that were each spiked with OTC at a level of 100 μg kg^{-1}, equivalent to the MRL of OTC in milk. The obtained trueness, expressed as the percent of recovery was 85, 90 and 60 for milk, whey and cheese, respectively. The limit of quantification (LOQ) was defined as the lowest concentration or mass of the analyte that has been validated with acceptable accuracy, by applying the complete analytical method. The calculated LOQ was 10 μg kg^{-1} for milk and whey, and 40 μg kg^{-1} for cheese. The repeatability, expressed as the relative standard deviation "within-lab" repeatability (RSDwr; %), was 14% for milk and whey and 16% for cheese. The extended uncertainty, as percentage, was 11% for milk, 28% for whey and 27% for cheese.

2.6. Statistical analysis

The data for measured variables (mass balance and cheese composition) were analysed using a randomised complete block design, which incorporated control and OTC spiked-cheese samples (milk, whey and cheese) and 6 blocks (replicate samples obtained during the 6 replicate cheese-making trials). Analysis of variance (ANOVA) was carried out using a general linear model (GLM) procedure of Statgraphics Centurion XVI for Windows software package (version 16.2.04; Statpoint Technologies, Inc. Warrenton, Virginia, VA, USA) and the effects of treatment and replicate on each response variable were determined. Tukey's multiple-comparison test was used for paired comparison of treatment means and the level of significance was determined at $P < 0.05$.

3. Results and discussion

3.1. Cheese-making process

Spiking of milk with OTC at the MRL level (100 μg kg^{-1}) produced a delay in the acidification of milk by starter culture during cheesemaking. The time required to reach pH 5.60, when syneresis and acidification were considered to be complete, was delayed by about 60 min in the MRL OTC cheese, compared with the control cheese. Our findings are consistent with those of other authors indicating possible adverse technological effects, during the cheese manufacture, due to the presence of oxytetracycline in the milk, even though at MRL level. Effects of similar concentrations of β-lactams on acid development are reported, both in sheep milk yoghurt (Berruga, Molina, Novés, Román, & Molina, 2007; Novés et al., 2015) and in pressed ewes' milk cheese (Berruga et al., 2008).

The mass balance (weights of sweet whey, draining whey and cheese, as proportions of the weight of cheese milk) and recoveries of fat and protein in the 1-day-old cheese are reported in Table 1.

The different acidification rates of control and MRL OTC curds during cheese manufacture had no effect ($P < 0.05$) on the overall release of whey, as can be seen from the similar weights of sweet whey, draining whey and cheese (Table 1). In fact, as can be seen in Table 1, no differences were noticed, in compared milks, as concern the distribution of products derived from the cheese-making process (sweet whey, draining whey, cheese). Additionally, there was

Table 1
Mass balance and cheese component recovery for cheese made from control milk or milk spiked with oxytetracycline (100 μg kg^{-1}).[a]

Mass balance and recovery	Cheese	
	Control	MRL OTC
Mass balance		
Sweet whey	74.2 ± 0.6	74 ± 1
Draining whey	7.3 ± 0.7	7.2 ± 0.5
1-day cheese	16 ± 2	16 ± 2
Recovery		
Fat	89 ± 2	88 ± 1
Protein	74 ± 3	74 ± 3

[a] Values (%, w/w) are means ± standard deviation (n = 6 replicate cheese-making trials); no significant differences ($P < 0.05$) were found between control cheese and cheese made from milk spiked with oxytetracycline (100 μg kg^{-1}; MRL OTC). Mass balance defined as outputs (sweet whey, draining whey, cheese) as a percentage of milk total weight; recovery is in 1-day cheese, expressed as weight of fat and protein in cheese as a percentage of fat and protein in milk, respectively.

no difference in the recovery of fat or protein ($P < 0.05$) between the control and MRL OTC treatments, probably because the presence of OTC in the milk did not alter its coagulability and, therefore, the behaviour of the coagulum during the curd-cutting stage. The cheese yield and recoveries of fat and protein (Table 1, 1-day-old cheese) were typical of those usually found for hard uncooked pecorino cheeses (Pirisi & Pes, 2011).

Table 2 shows the composition and soluble N levels of both 1- and 90-day-old control and MRL OTC cheese. It is interesting that despite the difference in the acidification rates between control and MRL OTC cheese during manufacture, the pH of 1-day-old cheeses did not differ. The moisture content of the 1-day-old OTC cheese was significantly higher than that of the corresponding control cheese. The higher moisture of the former cheese may have been associated with the slower acidification during manufacture. The presence of OTC in milk did not affect the other chemical parameters investigated (Table 2).

There was no difference in the composition or soluble N levels ($P > 0.05$) between the MRL OTC and control cheeses at 90 d (Table 2) The initial difference in moisture disappeared with ripening. The higher moisture content in the 1-day MRL OTC cheese, compared with the control cheese, may have resulted in a different behaviour during the salting phase, leading to a higher absorption of salt which, although not significant, probably induced a greater loss of moisture from the MRL OTC cheese during the initial phase of maturation, thus inducing a levelling of this parameter during ripening. The presence of OTC in cheese matrix did not affect the proteolysis (SN TN^{-1}, TCA-SN TN^{-1}, PTA-SN TN^{-1}), which was similar in control and MRL OTC cheese (Table 2). This outcome may be due to a similar availability of proteolytic enzymes of endogenous and bacterial origin in the two kinds of cheese.

The presence of the OTC residues in cheese did not affect the evolution of the observed biochemical parameters during cheese ripening. Indeed, a decrease in pH value, a reduction in moisture content, due to the normal loss of water, with a consequently increase in salt content, and an enhancement of proteolytic indices can be highlighted in both types of cheeses.

Table 2
Composition of cheese made from control milk or milk spiked with oxytetracycline.[a]

Parameter	1-day cheese		90-day cheese	
	Control	MRL OTC	Control	MRL OTC
pH	5.30 ± 0.08	5.27 ± 0.05	5.1 ± 0.1	5.2 ± 0.1
Moisture (%, w/w)	42.7 ± 0.7	43.9 ± 0.3	34 ± 3	33 ± 3
FDM (%, w/w)	50 ± 2	50 ± 1	49 ± 3	49 ± 2
PDM (%, w/w)	43 ± 1	42.8 ± 0.5	41.7 ± 0.8	42 ± 0.9
SN (%TN, w/w)	6 ± 1	7.4 ± 0.6	23.5 ± 0.4	23 ± 1
TCA-SN (%TN, w/w)	3.4 ± 0.4	3.2 ± 0.4	16 ± 2	16 ± 3
PTA-SN (%TN, w/w)	1.23 ± 0.02	1.2 ± 0.1	10 ± 4	9 ± 3
Ash (%, w/w)	2.5 ± 0.2	2.6 ± 0.2	4.3 ± 0.2	4.3 ± 0.4
Salt in moisture (%)			4 ± 2	5 ± 2

[a] Values are means ± standard deviation (n = 6 different batches for each treatment); values were not significantly different between control cheese and cheeses made from milk spiked with oxytetracycline (100 μg kg^{-1}; MRL OTC) with the exception of moisture in the 1-day cheeses, indicated by different superscript letters ($P < 0.05$). Abbrevations are: FDM, fat in dry matter; PDM, protein in dry matter; TN, total nitrogen; SN, nitrogen soluble in water; TCA-SN, nitrogen soluble in 12% trichloroacetic acid; PTA-SN, nitrogen soluble in 10% phosphotungstic acid.

3.2. Distribution of OTC in milk components

The distribution, between wheys (sweet and draining) and 1-day cheese, of the OTC added in milk is reported in Table 3.

About 60% of the total OTC amount added in the milk was recovered in cheese matrix, ~20% in the sweet whey and ~3% in the draining whey. Recently, Hakk et al. (2016) studied the distribution of OTC and other antibiotic molecules between skim milk and milk fraction in spiked whole cow milk, reporting that OTC was mainly recovered on skim milk due to its high hydrophilicity. Thus, it was expected to find the highest concentration of this molecule in whey fractions. Despite that, our results show that the concentration factor of the OTC molecule, from milk to 1-day cheese (3.8 ± 0.3), was comparable with those of fat (5.2 ± 0.4) and protein (4.6 ± 0.2). This suggests an interaction between OTC and the casein matrix of the curd. In support of this hypothesis it is known, in fact, that OTC binds to the animal proteins (Adetunji, 2011; Posyniak, Zmudzki, Semeniuk, Niedzielska, & Ellis, 1999) and presents a very high affinity for Ca^{2+} and Mg^{2+} (Arias et al., 2007; Comunian, Paba, Dupré, Daga, & Scintu, 2010; Martin, 1979). Further studies are needed to better clarify the nature of these interactions and verify the degree of association of the molecule with the main components of the curd.

A further amount of OTC (17%), was not detected. This result maybe, in our opinion, due to the contribution of the uncertainty of the adopted analytical method. In fact, no epimers and anhydro-derivatives (α-apo-OTC and β-apo-OTC) of OTC were detected, neither in the cheese, nor in the whey samples.

Interestingly, the OTC content in 90-day-old ripened cheese (321 ± 29 μg kg^{-1}) was significantly lower ($P < 0.05$) than that in the 1-day-old cheese (388 ± 11 μg kg^{-1}), denoting a reduction of 17 ± 8% during ripening. To explain its reduction a partial degradation of the molecule, during cheese ripening, can be assumed. However, this hypothesis needs to be investigated further in order to identify the degradation products and the mechanism of degradation of OTC during cheese maturation.

The recommended daily intakes of milk and cheese, as reported in food-based dietary guidelines (FBDG), vary significantly with country (FAO, 2016). Based on FAOSTAT data (FAO, 2015) for Europe,

Table 3
Distribution of oxytetracycline in milk, wheys and cheese.[a]

Component	Oxytetracycline	
	Partition (%)	Concentration (μg kg^{-1})
Milk	100	103 ± 9
1-Day cheese before salting	61 ± 5	388 ± 11
Sweet whey	21 ± 2	29 ± 4
Draining whey	2.8 ± 0.2	40 ± 2
90-day cheese		321 ± 29

[a] Values are means ± standard deviation (n = 6 different batches); partition of oxytetracycline added to milk in the resultant cheese, sweet whey and draining is expressed as a percentage of that added to milk.

a daily supply per person of about 250 mL of milk and 37 g of cheese are assumed. Further, the Joint FAO/WHO Expert Committee on Food (JECFA, 2002) recommends an acceptable daily intake (ADI) of 0–30 μg kg^{-1} of body weight for OTC. Based on the findings of the current study, we calculated the estimated daily intake (EDI) of oxytetracycline, assuming a regular intake of both MRL-contaminated milk and the cheese (i.e., 250 mL of milk and 37 g of cheese per person per day). Under these conditions the total intake of OTC would be 37 μg day^{-1} $person^{-1}$, which represents the 2.1% of the ADI for a consumer of about 60 kg body weight. As indicated by several authors, this value would lead to a not negligible risk (Aalipour, Mirlohi, Jalali, & Azadbakht, 2015; Vragović, Bažulić, & Njari, 2011), since dairy products are not the only possible source of antibiotics in human diet. However, exposure assessments indicated that processed bulk milk at the dairy plants in the European Union is unlikely to exceed MRL (EMEA, 2002) because of the effect of variability factors among which dilution with milk from untreated animals.

4. Conclusions

Cheese produced from milk spiked with the MRL of OTC showed a delay in acidification (pH reduction) during manufacture. Consequently, the moisture content in 1-day cheese was higher in the OTC cheese than in the control one. The initial difference in moisture disappeared during ripening, and the two types of cheeses had similar composition and soluble N levels at 1 and 90 d. Similarly, spiking of milk with the MRL of OTC did not significantly affect mass balance or the recovery of fat and protein from milk to cheese. Further studies, aimed to better highlight the effect of OTC at MRL level on starter cultures, may help to clearly understand the influence of this drug on lactic acid bacteria metabolism and assess the risk of adverse technological effects during the cheese manufacture. Most (60%) of the OTC added to whole raw ovine milk at the MRL level, was recovered in the 1-day-old cheese. The concentration of OTC decreased in the cheese during the 90 d ripening period, suggesting its partial degradation; the degradation products produced and the mechanism of degradation require further investigation. The hypothetical, though unlikely, simultaneous daily intake, at the amounts indicated in the dietary guidelines, of both MRL contaminated milk and derived cheeses, would be not negligible and could lead to an intake of OTC that contributes to the ADI value.

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6 Chapter III: Heat treatment of oxytetracycline spiked ovine milk: fate of the molecule and technological implications

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Abstract: A few studies have been published on the distribution of oxytetracyline (OTC) residues present in milk among cheese, whey, and milk protein fractions, throughout cheese-making, most of them, focused on the effect of pasteurization and Ultra High Temperature (UHT) treatments, and carried out on cow milk. The aim of this study was to investigate the fate of oxytetracycline residues in spiked ovine milk, at MRL (maximum residue limit) and half MRL, after thermization, throughout the cheese-making and in ripened cheeses. The antibiotic recovery and partition from milk into whey and cheese were assessed by liquid chromatography-high resolution mass spectrometry (LC-HRMS). Starter and non-starter microflora development was monitored by viable plate counts. Milk thermization did not affect OTC recovery, partition and cheese chemical composition. On a dry matter basis, an OTC reduction between 15 and 19% was calculated in 60-day cheese, at MRL and half MRL, respectively. OTC caused a dose-dependent difference in the time required to reach pH 5.60, which was significantly higher ($P < 0.05$) at MRL level (406 ± 2 min) compared to half MRL level and the control (363 ± 30 and 328 ± 24 min, respectively). This allowed coliform bacteria to reach 6 log CFU g-1.

HEAT TREATMENT OF OXYTETRACYCLINE SPIKED OVINE MILK: FATE OF THE MOLECULE AND TECHNOLOGICAL IMPLICATIONS

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Abstract

A few studies have been published on the distribution of oxytetracyline (OTC) residues present in milk among cheese, whey, and milk protein fractions, throughout cheese-making, most of them, focused on the effect of pasteurization and Ultra High Temperature (UHT) treatments, and carried out on cow milk. The aim of this study was to investigate the fate of oxytetracycline residues in spiked ovine milk, at MRL (maximum residue limit) and half MRL, after thermisation, throughout the cheese-making and in ripened cheeses. The antibiotic recovery and partition from milk into whey and cheese were assessed by liquid chromatography-high resolution mass spectrometry (LC-HRMS). Starter and non-starter microflora development was monitored by viable plate counts. Milk thermisation did not affect OTC recovery, partition and cheese chemical composition. On a dry matter basis, an OTC reduction between 15 and 19% was calculated in 60-day cheese, at MRL and half MRL, respectively. OTC caused a dose-dependent difference in the time required to reach pH 5.60, which was significantly higher ($P < 0.05$) at MRL level (406 ± 2 min) compared to half MRL level and the control (363 ± 30 and 328 ± 24 min, respectively). This allowed coliform bacteria to reach 6 log CFU g^{-1}.

Keywords

oxytetracycline, ovine milk, thermisation, antibiotic residues, starter LABs

1. Introduction

Antimicrobial agents are an important tool to treat and prevent diseases and as metaphylaxis in food producing animals. However, their presence as residues in foods of animal origin may constitute a risk to public health, due to the possible induction of allergic reactions in consumers (Adetunji, 2011), and to their potential contribution to the development of antimicrobial resistance phenomena (AMR) (EFSA, 2017).

Antibiotics residues occur in milk mainly as a result of incorrect veterinary treatments, such as the adoption of uncorrected withdrawal periods, or the unregulated use of extra label molecules (Beyene, 2016). Sometimes, these wrong practices can lead to overcome the maximum residue limit (MRL) adopted for milk by the international authorities in order to protect the consumers.

As observed by many authors, antibiotics residues, even when present in milk below the MRL level, can negatively affect the technological process of milk-derived products (Berruga, Beltrán Martínez, Novés, Molina, & Molina, 2011). Microorganisms continuously exposed to antibiotics at sub-inhibitory concentrations can undergo a specific transcriptional change that will modify their metabolic activity (Broszat & Grohmann, 2014). For instance, residues of OTC in yogurt and cheese may cause a significant delay in the pH decrease rate and defects in the development of lactic acid bacteria (Berruga, Battacone, Molina, Román, & Molina, 2008; Cabizza et al., 2017; Suhren, 1998), and interfere with the coagulation and ripening phases affecting the sensory properties of cheeses (Berruga, Molina, Althaus, & Molina, 2016; Nagel et al., 2009).

A few studies have been published on the distribution of antibiotics residues among cheese, whey, and milk protein fractions, during cheese-making. Shappell and co-workers (2017), working in tubes, at a laboratory scale, found that only 14% of oxytetracycline (OTC) added to the milk was recovered in the curd, and, based on lipophilicity characteristics of various type of animal drugs molecules, provided an empirical model for predicting their distribution between cow skim milk curd, whey and associations with proteins. However, the actual process conditions and the cheese-

60 making technology applied should be taken into account, since they could affect the molecules
61 distribution. Indeed, Cabizza and co-workers (2017), who studied the transfer of oxytetracycline
62 added in ovine raw milk at MRL level (100 μg kg^{-1}), showed that the cheese was able to retain most
63 of the molecule added to the milk and the presence of the molecule produced a delay in the
64 acidification.
65 Moreover, the impact on the microbiota is even less studied and, to date, an investigation assessing
66 the effect of low-dosage antibiotics on starter lactic acid bacteria development during cheese-
67 making lacks. In addition, most of the ovine milk undergoes thermisation or pasteurization before
68 cheese-making in order to contrast the development of pathogen and spoilage bacteria, preventing
69 possible technological defects in cheese. Therefore, the ability of these treatments to reduce the
70 antibiotics concentrations should be also investigated. Some authors report a thermal-dependent
71 reduction in OTC content of different contaminated matrices (Hassani, Lázaro, Pérez, Condón, &
72 Pagán, 2008; Kellnerová, Navrátilová, & Borkovcová, 2015; Shahani, 1958). The available studies
73 on the effect of milk thermal treatments on OTC degradation were mainly carried out on cow milk
74 and focused on pasteurization, while no data are available on ovine milk and the effect of
75 thermisation. Shahani (1958) reported a 23.6% degradation of OTC, in milk spiked at 320-900 μg
76 L^{-1}, after a low time low temperature (LTLT) treatment (61.7 °C, 30 min). Kellnerová et al. (2015)
77 reported a 15.3% degradation of OTC in 1.5 MRL spiked milk, after high pasteurization treatment
78 (85 °C, 3 s), while an ultra-high-temperature sterilization (UHT) was shown to be effective to
79 completely degrade the OTC spiked in McIlvaine buffer (Hassani et al., 2008). However, most of
80 these studies were focused on hardly comparable OTC concentrations ranges and time-temperature
81 combinations, performed in laboratory conditions, and often on cow milk or other matrices.
82 Therefore, the aim of this work was to assess if the thermisation treatment reduces oxytetracycline
83 content in spiked ovine milk, and to investigate the effect of OTC residues on the starter culture
84 development and cheese composition. The antibiotic recovery and partition, during cheese-making

an ripening process, from milk into whey and cheese, were also compared to results reported by Cabizza et al. (2017) for raw milk.

2. Material and methods

2.1. Chemicals and reagents

Oxytetracycline hydrochloride (OTC, purity ≥96.7%) was purchased from Sigma-Aldrich (St. Louis, MO, USA); 4-epi-oxytetracycline (EOTC, purity ≥97%), α-apo-oxytetracycline (α-apo-OTC) and β-apo-oxytetracycline (β-apo-OTC) were purchased from Acros Organics (Geel, Belgium). All solvents used were liquid chromatography-mass spectrometry (LC-MS) grade from Carlo Erba (Milan, Italy), and LC-MS grade water was produced with an Advantage System (Millipore, Billerica, MA, USA). Stock and spiking solutions were prepared as previously described (Cabizza et al., 2017).

2.2 Cheese-making process

Ovine milk was collected from an experimental flock of Sarda breed sheep at AGRIS Sardegna Research Agency (Olmedo, Sassari, Italy). Sheep were in good health and did not undergo antibiotics treatments. The milk was divided into 3 vats of 12 kg each. The first one was the control (not spiked), the second and the third one were spiked with OTC in order to obtain milk at half MRL (50 μg kg^{-1}) and at MRL level (100 μg kg^{-1}), respectively. Subsequently, raw milk was heated up to 63°C, in each vat, and immediately cooled until 38°C, obtaining the heat penetration curve reported in Fig. 1.

Then, the starter culture was inoculated (6 log CFU g^{-1}, CHOOZIT® Su Casu LYO, Danisco, Denmark) and calf rennet added (500 IMCU kg^{-1}, Bellucci, Italy), following the cheese-making

process described in Fig. 2, in order to obtain uncooked, hard, pecorino cheeses ripened until 60 days. The experiment was replicated three times in a short period (3 consecutive weeks, in January 2016) to minimize the possible effect of milk composition.

Data from the heat penetration curve obtained in the present work were used to calculate the cook value or C value (Awuah, Ramaswamy, & Economides, 2007), as reported in Equation 1:

$$C = \int_0^t 10^{(T-T_r)/z_r}\, dt \quad (1)$$

where t is the duration of treatment (min), T is the temperature, T_r is the reference temperature and z_r is the reference temperature resistance coefficient. The trapezoidal rule was used for integration. The same procedure was used to estimate C values of thermal treatment performed by Shahani (1958) and Kellnerová et al. (2015).

2.3. Milk and cheese composition, mass balance and component recoveries (fat and protein)

The parameters of milk composition were evaluated by using MilkoScan FT+ (Foss, Hillerød, Denmark) as described in ISO 9622 (ISO, 2013). pH was measured by pH meter (Knick 911, Knick, Berlin, Germany), equipped with a InLab® Solids electrode (Mettler Toledo, Ohio, USA). The pH of cheeses was measured every 60 minutes until 300 minutes and then every 30 minutes until pH 5.5. Total solids were measured according to the ISO method 6731 (ISO, 2010). Cheeses, at 1 and 60 days of ripening, were analysed for many parameters: pH (pH meter Crison Basic 20+, equipped with a 5232 Puncture electrode); dry matter in accordance with ISO 5534 (ISO, 2004); fat (Soxhlet, 1879), lipolysis index (Nuñez, García-Aser, Rodríguez-Martin, Medina, & Gaya, 1986); protein in accordance with ISO 8968-1(ISO, 2014); pH 4.6-soluble N, 12% trichloroacetic acid soluble N, 5% phosphotungstic acid-soluble N as described by Gripon, Desmazeaud, Le Bars, and Bergere (1975); total ash by gravimetric analysis after ashing the sample at 550 °C for 12 h according to ISO 27:1964 (ISO, 1964); NaCl according to ISO 5943:2006 (ISO, 2006). Each

133 analysis was performed in duplicate. Mass balance was expressed as indicated in Cabizza et al.
134 (2017). The component recoveries (fat and protein) in 1-day cheese were calculated as described in
135 Guinee, Mulholland, Kelly, and Callaghan (2007).

136

137 *2.4. Measurement of OTC levels in milk, whey and cheese*

138 *2.4.1. Sample extraction and solid phase extraction parameters*

139 Milk and whey samples (2.0 ± 0.1 g) were weighed into a 25 mL centrifuge tube; 10 mL of Mc
140 Ilvaine EDTA buffer (pH 4) were then added and mixture was agitated using a vortex (Classic
141 Advanced Vortex Mixer, Velp Scientifica, Usmate Velate, MB) and centrifuged at 12800 x g for 10
142 min at 0 °C (Centrifuge 5810R, Eppendorf, Hamburg, Germany). The supernatant was purified
143 using a Gilson automatic sample processor ASPEC on 3 mL OASIS HLB SPE cartridges,
144 previously conditioned with 3 mL MeOH and 2 mL H_2O. The sample (5 mL) was then loaded on
145 the cartridge, subsequently washed with 1.5 mL H_2O:MeOH (95:5, v/v). The analytes were eluted
146 with 2 mL MeOH into conical-bottom centrifuge tubes. The eluates were then evaporated near to
147 dryness under a gentle stream of nitrogen, reconstituted into 2 mL 14% MeOH (in water), and
148 filtered on a 0.20 mm Hydrophilic PTFE Millex Samplicity filter (Millipore).
149 The extraction of OTC from the cheese involved weighing a sample of the grated cheese (1.0 ± 0.1
150 g) into a 7 mL Precellys tube (Bertin Technologies, Aix-en-Provence, France), extracting with 5 mL
151 of Mc Ilvaine EDTA buffer (pH 4) at 2000 x g for 20 s using a Precellys 24 System homogenizer
152 (Bertin Technologies), centrifuging the resultant homogenate at 12800 x g for 10 min at 0 °C, and
153 transferring the supernatant into a glass centrifuge tube. The extraction process was repeated and
154 the combined extracts were purified, as described above for milk and whey samples.
155

156

157 *2.4.2. Liquid chromatography-tandem mass spectrometry analysis*

158 Liquid chromatography-tandem high-resolution mass spectrometry (LC-HRMS) analysis was
159 performed using an UPLC Ultimate 3000 (Thermo Fisher-Dionex San Jose, CA, USA) system was
160 coupled by a HESI-II electrospray source to a Q-Exactive Orbitrap™-based mass spectrometer (all
161 Thermo Scientific, San Jose, CA, USA). Chromatographic separation was performed on Waters
162 Acquity BEH C18 (100 mm x 2.1 mm, 1.7 µm particles) column preceded by a guard column BEH
163 Shield RP18 (5 mm x 2.1 mm, 1.7 µm particles), gradient elution with H_2O with 0.1% (v/v) formic
164 acid (A) and MeCN (B). The injection volume was 2 µL, the flow rate was 0.400 mL min^{-1}, and the
165 adopted gradient varied the % of A to B as follows: T 0.0 min, 80%; T 0.50 min, 80%; T 5 min, 0
166 %; T 6.50 min, 0%; T 6.60 min, 80%; T 8.00 min, 80%; the target column temperature was 45 °C.
167 Q-Orbitrap HRMS with HESI-II electrospray source was operated in positive mode.
168 The following ionization parameters were applied: electrospray voltage 4 kV for positive mode,
169 capillary temperature 300 °C, Aux gas heater temp 330 °C, sheath gas (N_2) 40 arbitrary units (arb),
170 auxiliary gas (N_2) 10 (arb), and S-Lens RF level at 50 (arb). LTQ Velos ESI positive-ion calibration
171 solutions (Thermo Scientific, San Jose, CA).

172 The acquisition was achieved in full scan/dd-MS^2 mode.

173 Full MS mode: mass range m/z 150–500, resolution of 70000 FWHM (m/z 200), AGC target 3.0E6,
174 maximum injection time of 100 ms; dd-MS^2 mode: resolution of 17500 FWHM (m/z 200), AGC
175 target was set at 1.0E5 ions, maximum injection time of 75 ms, isolation window of m/z 2.0.
176 The obtained retention time (RT) of analytes of OTC, EOTC, α-apo-OTC and β-apo-OTC were
177 2.56, 2.21, 3.04 min, respectively. MS monitored masses in HESI+ mode (m/z) were: OTC,
178 precursor 461.1555, fragments 426.1 and 337.1; EOTC, precursor 461.1555, fragments 426.1 and
179 444.1; α-apo-OTC and β-apo-OTC, precursor 443.1449, fragment 426.1 (fragment ions are used
180 only for qualitative purposes

181 *2.4.3. Method validation*

182 An in-house validation protocol was performed out to evaluate the performances of the method, in
183 order to ensure adequate identification, confirmation and quantification of OTC. The method was
184 validated in milk, whey and cheese by the criteria described below.

185 The selectivity and specificity were assessed by analysing 6 blank samples from each matrix. The
186 absence of background peaks, above a signal-to-noise ratio of 3, at the retention time of OTC
187 showed that the method was free of endogenous interferences. The trueness was determined by
188 analysing 6 separate samples of milk, whey and cheese that were each spiked with OTC at a level of
189 100 μg kg^{-1}, equivalent to the MRL of OTC in milk. The obtained trueness, expressed as the percent
190 of recovery was 102 and 80 for milk, whey and cheese, respectively. The limit of quantification
191 (LOQ) was defined as the lowest concentration or mass of the analyte that has been validated with
192 acceptable accuracy, by applying the complete analytical method. The calculated LOQ was 1 μg kg^{-1}
193 for milk and whey, and 10 μg kg^{-1} for cheese. The repeatability, expressed as the relative standard
194 deviation "within-lab" repeatability (RSDwr; %), was 9% for milk and whey and 10% for cheese.
195 The extended uncertainty, as percentage, was 22% for milk and whey and 25% for cheese.
196

197 *2.5 Microbiological analysis*

198 Samples of thermised milk, before and after the inoculum of the starter culture, 1 and 60 days
199 ripened cheeses were prepared according to the IDF standard 122C (1996). Viable counts were
200 carried out to enumerate: starter lactic acid bacteria (SLAB), i.e. thermophilic cocci and lactobacilli,
201 on M17 agar and MRS agar media (Microbiol, Cagliari, Italy), respectively, incubated at 45 °C, in
202 anaerobiosis for 72 h; non-starter lactic acid bacteria (NSLAB), i.e. mesophilic lactobacilli, on FH
203 agar medium (Isolini et al., 1990), incubated at 37 °C, in anaerobiosis for 72 h; Enterococci on
204 KAA medium (Microbiol), incubated at 42 °C, in aerobiosis, for 18-24 h; coliform bacteria, on
205 VRBA MUG (Microbiol), incubated at 37 °C, in aerobiosis for 18-24 h.

206

207 *2.6 Statistical analysis*

208 Statistical analysis was performed by Statgraphics Centurion XVI for Windows software package
209 (version 16.2.04; Statpoint Technologies, Inc. Warrenton, Virginia, VA, USA). Analysis of
210 variance (ANOVA) was carried out using the general linear model (GLM) to determine the effects
211 of treatment and replicate on each response variable. Tukey's multiple comparison test was used for
212 paired comparison of treatment means ($P < 0.05$).

213

214 **3. Results and discussion**

215 *3.1 Milk and cheese composition, mass balance and components recoveries*

216 The physical-chemical characteristics of the milk (mean ± s.d.) were: total solids 15.1 ± 0.1;
217 protein, 4.8 ± 0.1; fat, 4.9 ± 0.1; casein, 3.5 ± 0.1; and lactose, 4.8 ± 0.1 (g 100 g^{-1} of milk). The pH
218 was 6.7 ± 0.1.
219 OTC spiked milk (half MRL and MRL) showed a dose-dependent delay during the early
220 acidification phase (Fig 3), until reaching pH 5.60 (35 ± 11 and 78 ± 26 min, at half MRL and MRL
221 levels). In fact, the time required to reach pH 5.60, was significantly higher ($P < 0.05$) at MRL level
222 (406 ± 2 min) compared to half MRL level and the control (363 ± 30 and 328 ± 24 min,
223 respectively).
224 These results are in accordance with those previously noticed for raw milk by Cabizza et al. (2017),
225 as milk thermisation did not affect OTC activity and its influence on starter culture acidification
226 performance.
227 The delay in acidification profiles did not result in a different mass balance (weights of sweet whey,
228 draining whey and cheese, as proportions of total processed milk weight) between control and
229 experiments (Table 1). Control and OTC (both half MRL and MRL) cheese yields, calculated as

230 ratio between produced cheese at 1-day and milk used for cheese-making, were comparable.
231 Similarly, the same component recoveries in control and experimental cheeses were observed, as
232 already noticed for cheese obtained from raw milk (Cabizza et al., 2017). Furthermore, no
233 differences in pH, composition and soluble N levels, between control and experimental cheeses,
234 both at 1 and 60 days of ripening, were observed (Table 2). The chemical characteristics of 60-day
235 ripened cheeses were close to those of Pecorino Sardo cheese (Pirisi, Comunian, Urgeghe, &
236 Scintu, 2011).
237
238 *3.2 Measurement of OTC levels in fractions*

239 The thermal treatment of spiked milk samples did not reduce the concentration of OTC both in half
240 MRL (47 ± 6 μg kg^{-1} and 42 ± 1 μg kg^{-1}, raw vs. thermised) and MRL (90 ± 11 μg kg^{-1} and 90 ± 13
241 μg kg^{-1}, raw vs. thermised) spiked milk samples. Using for OTC a z_{ref} value of 8.88 °C and a T_{ref} of
242 71.1 °C, as provided by Shahani (1958) for cow milk, in the pasteurization temperature range, we
243 obtained a C value of 1.64 ± 0.13. This value should be equivalent to about 84% of that estimated
244 (C value of 1.84) using the conditions applied (85 °C, 3 s) by Kellnerová et al. (2015), which
245 resulted in a 15.3% antibiotic drop. On this basis, a residual concentration of 36.4 ± 0.5 μg kg^{-1} and
246 78 ± 11 μg kg^{-1} should have been expected for samples spiked at half MRL and MRL, respectively.
247 However, the heat degradation of oxytetracycline is reported to be also dependent from the total
248 solid content of the milk, with a lower level of degradation as the solid content, particularly fat,
249 increases (Moats, 1999; Tian, Khalil, & Bayen, 2017). Ovine milk, whose total solid, especially fat,
250 content is higher than cow milk (Park, Ju, Ramos, & Haenlein, 2007), could have protected OTC
251 from degradation during the thermal treatment applied in this study. The adopted time-temperature
252 profiles in cheese-making practices usually are milder than that reported in the above-cited
253 literature, and the treatment is often performed at the plant by continuous systems. Further, as many
254 cheeses derived from milk undergone to thermisation, particularly those made from ovine milk

255 (Pirisi & Pes, 2011), it can expect that the thermal treatment of ovine milk, in cheese-making
256 conditions usually applied at dairy plant, should produce a poor or negligible degradation of OTC.
257 The distribution between wheys (sweet and draining) and cheese of the OTC added in milk is
258 reported in Table 3. The partition between the obtained fractions appeared to be independent from
259 the spike level, and was similar to that previously observed in the experiments performed with raw
260 milk (Cabizza et al., 2017), except for the cheese fraction, which was able to retain a greater amount
261 of the added molecule (about 80%) than the cheese made from raw milk (about 60%). The overall
262 molecule recovery, close to 100%, obtained in this study, could be ascribable to the better
263 performances provided by the adopted analytical method, involving LC-HRMS, indicating that the
264 unrecovered amount (17 %) obtained by Cabizza et al. (2017) could be attributable to the cheese.
265 The concentration of OTC in cheese, for both the studied levels, did not decrease with ripening,
266 (Table 3), differing from what previously observed for cheese obtained from raw milk (Cabizza et
267 al., 2017). Actually, in absence of degradation phenomena, an increase in concentration of OTC
268 should be expected, as for the other components, due to the reduction of moisture. However,
269 considering the concentration on a dry matter basis, it was possible to calculate a reduction of OTC
270 with ripening, from 413 ± 11 μg kg^{-1} to 335 ± 3 μg kg^{-1} of dry matter (19 ± 2%), and from 832 ± 57
271 μg kg^{-1} to 707 ± 25 μg kg^{-1} of dry matter (15 ± 4%), in half MRL and MRL cheeses, respectively.
272 Some consequences of the thermal treatment of milk, such as the modification of the enzymatic
273 pattern and the reduction of the redox potential (McSweeney & Fox, 2009) could have contributed
274 to the stability of OTC during ripening. In our opinion this could be a reason of the lower reduction
275 of OTC (about 50%) compared to that previously observed in raw milk cheese (Cabizza et al.,
276 2017), even if, in that case, cheese underwent to a longer ripening period.
277

278 *3.3 Microbiological analysis*

279 Results of viable counts, carried out to verify the effect of OTC on microflora development in 1-day
280 and 60-day cheeses, are reported in Table 4.
281 No significant differences were detected between viable counts of the microbial groups searched in
282 control and experimental thermised milk samples, both before and after the starter inoculum, whose
283 level was confirmed to be about 6 log CFU mL^{-1}. NSLAB, Enterococci and coliform bacteria
284 counts were < 1 log CFU g^{-1} in all the thermised milk samples analysed.
285 In 1-day cheese, SLAB (thermophilic lactobacilli and thermophilic streptococci) reached the same
286 average log CFU g^{-1} in control and experimental samples, despite a pronounced delay during the
287 acidification phase. Whereas, coliform bacteria counts were significantly higher ($P < 0.05$) in MRL
288 level, than in half MRL and control samples. This difference is attributable to the acidification
289 delay, which resulted in more favourable environmental growth conditions (higher pH in OTC
290 cheeses than in control ones) for coliform bacteria that were able to reach 6 log CFU g^{-1}. This is a
291 crucial issue to be taken into account, since high levels of coliform bacteria could have negative
292 technological implications during the cheese-making, resulting into cheese defects, as well as being
293 a safety concern for human health, particularly in short-ripened cheeses.
294 In 60-day cheese, thermophilic lactobacilli decreased about 1 log CFU g^{-1}, in all samples, while
295 thermophilic streptococci remained stable in both OTC spiked samples, and decreased about 1 log
296 CFU g^{-1} in the control, whose counts resulted significantly different ($P < 0.05$) from both OTC
297 samples. In fact, as reported by Broszat and Grohmann (2014), a higher ability to survive could be
298 acquired by microorganism as stress response to sub-inhibitory concentrations of antibiotics that
299 could modify their metabolic activity. As expected, NSLAB, whose important role during ripening
300 is well known, though were almost undetectable in 1-day cheese, increased in all samples, until 4
301 log CFU g^{-1}. Enterococci remained constant, while coliform bacteria decreased during ripening
302 (about 4 log CFU g^{-1}), showing no significant differences among counts of OTC (both MRL and
303 half MRL) and control 60-day cheeses.
304

4. Conclusions

The thermal treatment performed did not reduce the concentration of OTC, both in half MRL and MRL spiked ovine milk samples, probably because of a protective effect of the higher total solid content of the ovine milk, especially fat, compared to cow milk. No differences on mass balance, yields, chemical composition between experimental and control cheese-making processes, 1 and 60-day cheeses, were observed.

The LC-HRMS based method adopted allowed to obtain an overall recovery of the molecule spiked in milk close to 100%.

The OTC residues in cheese underwent a modest decrease during ripening. Thermisation may have caused a reduction of the redox potential and a selection of the enzymatic pattern, which contributed to the stability of the molecule, resulting in a lower decrease, compared to that observed for raw milk cheese, in a previous work.

The observed delay in acidification of thermised milk spiked with OTC, compared to an antibiotic free control, proved a dose-dependent influence of low levels OTC residues on the starter culture development and metabolism, which affected lactic acid production. However, analysing 1-day cheese samples no differences in pH and SLAB counts were detected, leading to assume a temporary OTC effect, limited to the early hours of the acidification phase that has to be further investigated.

Acknowledgments

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419

Tables

Table 1
Technological parameters

Technological parameters	Control	Half MRL OTC	MRL OTC
Time to reach pH 5.60 [a]	328 ± 24[B]	363 ± 30[B]	406 ± 2[A]
Mass balance [b]			
Sweet whey	76 ± 1	76.6 ± 0.2	76.4 ± 0.7
Draining whey	6.1 ± 0.4	6.0 ± 0.3	6.0 ± 0.2
1-day cheese	15.1 ± 0.1	15 ± 0.1	15.1 ± 0.5
Recovery (%) [c]			
Fat	84 ± 4	84 ± 3	84 ± 6
Protein	73 ± 1	73 ± 2	74 ± 2

[a] Time expressed in minutes during the acidification phase to reach pH 5.60.
[b] Values (g 100 g^{-1} of milk) are means ± standard deviation (n = 3).
Mass balance defined as outputs (sweet whey, draining whey, cheese) as a percentage of milk total weight.
[c] Recovery in 1-day cheese, defined as percentage of fat and protein weight in cheese on fat and protein weight in milk, respectively. Level of spike with oxytetracycline (OTC): Half MRL, 50 µg kg^{-1}; MRL, 100 µg kg^{-1}. Values are means ± standard deviation (n = 3); values within a row not sharing a common superscript letters were significantly different ($P < 0.05$).

Table 2
Physico-chemical parameters of 1-day and 60-day cheese [a]

Parameter	1-day cheese						60-day cheese					
	Control		Half MRL OTC		MRL OTC		Control		Half MRL OTC		MRL OTC	
pH	5.4	± 0.1	5.4	± 0.1	5.4	± 0.1	5.1	± 0.1	5.2	± 0.1	5.2	± 0.1
Moisture (g 100 g^{-1} of cheese)	45.0	± 0.4	45.2	± 0.6	45.1	± 0.9	33.9	± 0.8	33.9	± 0.8	34	± 1
FDM (g 100 g^{-1} DM)	50	± 1	50	± 1	49	± 2	49.8	± 0.5	50.0	± 0.2	49.9	± 0.3
PDM (g 100 g^{-1} DM)	41.7	± 0.7	42.1	± 0.3	42.3	± 0.3	40.5	± 0.4	41	± 1	41	± 1
SN (g 100 g^{-1} TN)	6.9	± 0.8	8	± 1	7	± 2	13.7	± 0.3	15	± 1	15	± 1
TCA-SN (g 100 g^{-1} TN)	2.7	± 0.6	2.6	± 0.6	2.6	± 0.6	8	± 1	8.9	± 0.6	8.6	± 0.9
PTA-SN (g 100 g^{-1} TN)	1.4	± 0.2	1.5	± 0.2	1.5	± 0.2	4.0	± 0.2	3.9	± 0.3	3.8	± 0.3
FFA (meq 100 g^{-1} fat)	1.0	± 0.3	1.3	± 0.2	1.0	± 0.2	1.8	± 0.1	2.1	± 0.8	1.3	± 0.4
Ash (g 100 g^{-1} of cheese)	2.7	± 0.3	3	± 0.2	3.3	± 1	5.2	± 0.4	4.9	± 0.7	5.2	± 0.2
Salt (g 100 g^{-1} of moisture)	–		–		–		6.2	± 0.7	6.4	± 0.7	6.5	± 1.1

[a] Values are means ± standard deviation (n = 3); Abbreviations are: OTC, oxytetracycline; MRL, maximum residue limit; FDM, fat in dry matter; PDM, protein in dry matter; TN, total nitrogen; SN, nitrogen soluble in water; TCA-SN, nitrogen soluble in 12 % trichloroacetic acid; PTA-SN, nitrogen soluble in 10 % phosphotungstic acid; FFA, free fat acids.

Table 3
Distribution of OTC in milk, wheys and cheese [a]

	Half MRL OTC	MRL OTC
OTC partition (%)		
Milk	100	100
1-day cheese before salting	81.4 ± 0.8	77 ± 8
Sweet whey	23 ± 9	20 ± 3
Draining whey	2.9 ± 0.5	2.2 ± 0.4
OTC Concentration (μg kg^{-1})		
Milk	41.8 ± 0.6	90 ± 13
1-day cheese before salting	226 ± 4	457 ± 34
Sweet whey	13 ± 5	24 ± 6
Draining whey	20 ± 2	33 ± 3
60-day cheese	222 ± 3	470 ± 18

[a] Values (%, μg kg^{-1}) are means ± standard deviation (n = 3); OTC partition (%) in component derived from milk, defined as percentage of absolute amount (μg) in 1-day cheese, sweet whey and draining whey on total absolute amount of OTC added in milk (50 μg kg^{-1}, Half MRL OTC; 100 μg kg^{-1}, MRL OTC).

12

Table 4
Thermised milk, thermised milk + starter, 1-day and 60-day cheese viable counts [a]

Parameter	Thermised milk				Thermised milk + starter			
	Control	Half MRL OTC	MRL OTC		Control	Half MRL OTC	MRL OTC	
Thermophilic cocci	1.1 ± 0.5	1.37 ± 0.05	1.42 ± 0.14		5.6 ± 0.1	5.9 ± 0.1	5.8 ± 0.1	
Thermophilic lactobacilli	0.6 ± 0.6	1.0 ± 0.2	1.0 ± 0.1		4.23 ± 0.08	4.49 ± 0.05	4.3 ± 0.1	
Mesophilic lactobacilli	0 ± 0	0.1 ± 0.1	0.3 ± 0.4		0.1 ± 0.2	0 ± 0	0 ±	
Enterococci	0.2 ± 0.3	0 ± 0	0.6 ± 0.6		0 ± 0	1 ± 1	0.4 ± 0.7	
Coliform bacteria	0.3 ± 0.5	0.4 ± 0.5	0 ± 0		0.3 ± 0.5	0.3 ± 0.6	0.1 ± 0.2	
Parameter	1-day cheese				60-day cheese			
	Control	Half MRL OTC	MRL OTC		Control	Half MRL OTC	MRL OTC	
Thermophilic cocci	8.6 ± 0.4	8.6 ± 0.4	8.8 ± 0.5		7.7 ± 0.3[b]	8.6 ± 0.4[a]	8.6 ± 0.4[a]	
Thermophilic lactobacilli	5.3 ± 0.3	5.6 ± 0.2	5.5 ± 0.1		4.6 ± 0.4	4.8 ± 0.4	4.7 ± 0.2	
Mesophilic lactobacilli	0.2 ± 0.4	0.1 ± 0.1	0.00 ± 0.00		4.7 ± 0.2	4 ± 2	5 ± 2	
Enterococci	3.5 ± 0.6	4.4 ± 0.4	4 ± 1		3.6 ± 0.6	4.1 ± 0.5	4 ± 1	
Coliform bacteria	5.1 ± 0.2[b]	4.8 ± 0.5[b]	6.10 ± 0.09[a]		1.7 ± 0.5	1 ± 1	2 ± 3	

[a] Values (log CFU mL^{-1} for milk and log CFU g^{-1} for cheese) are means ± standard deviation (n = 3). Values within a row not sharing a common superscript letters were significantly different ($P < 0.05$).

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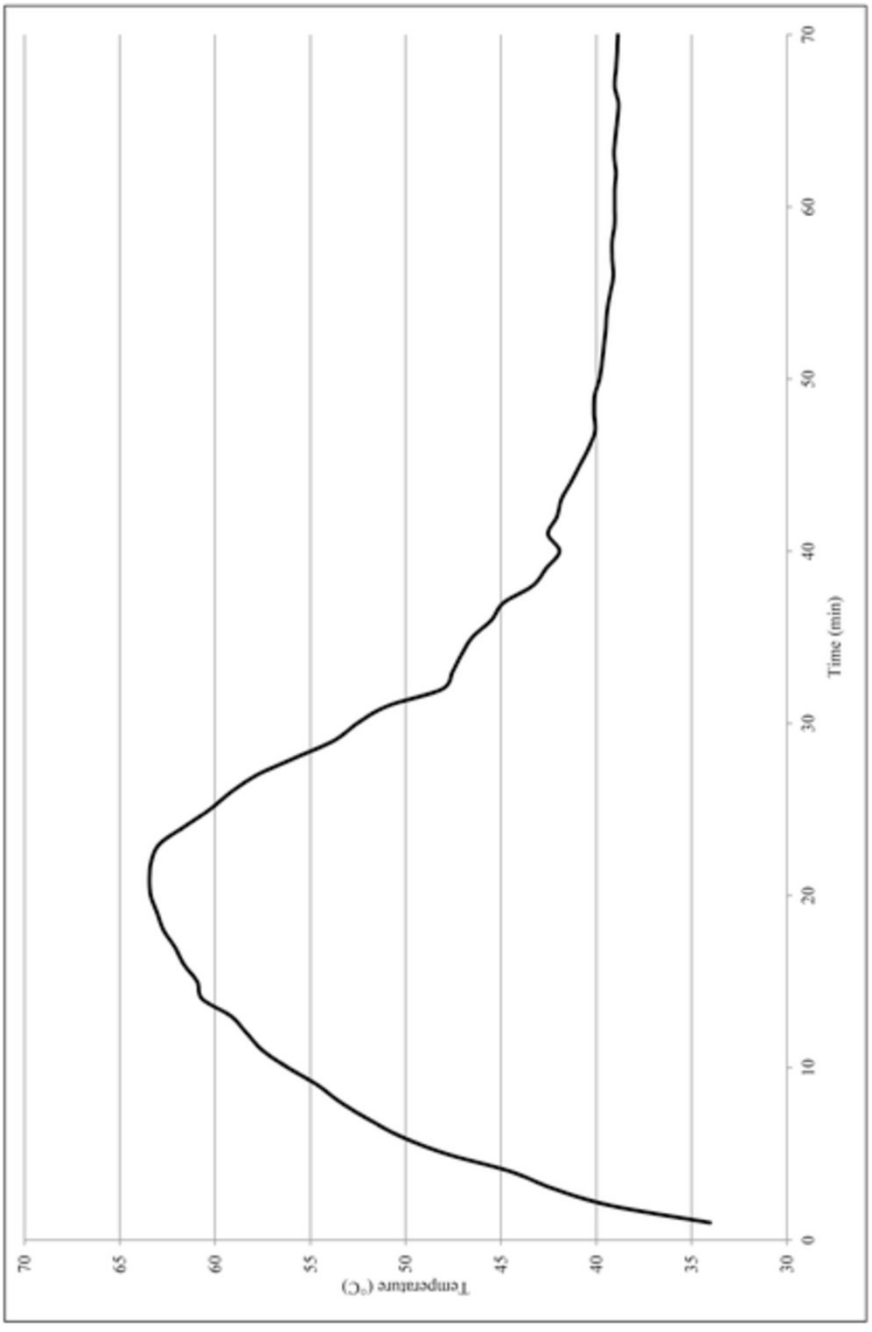

Figure 1
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Figure 2
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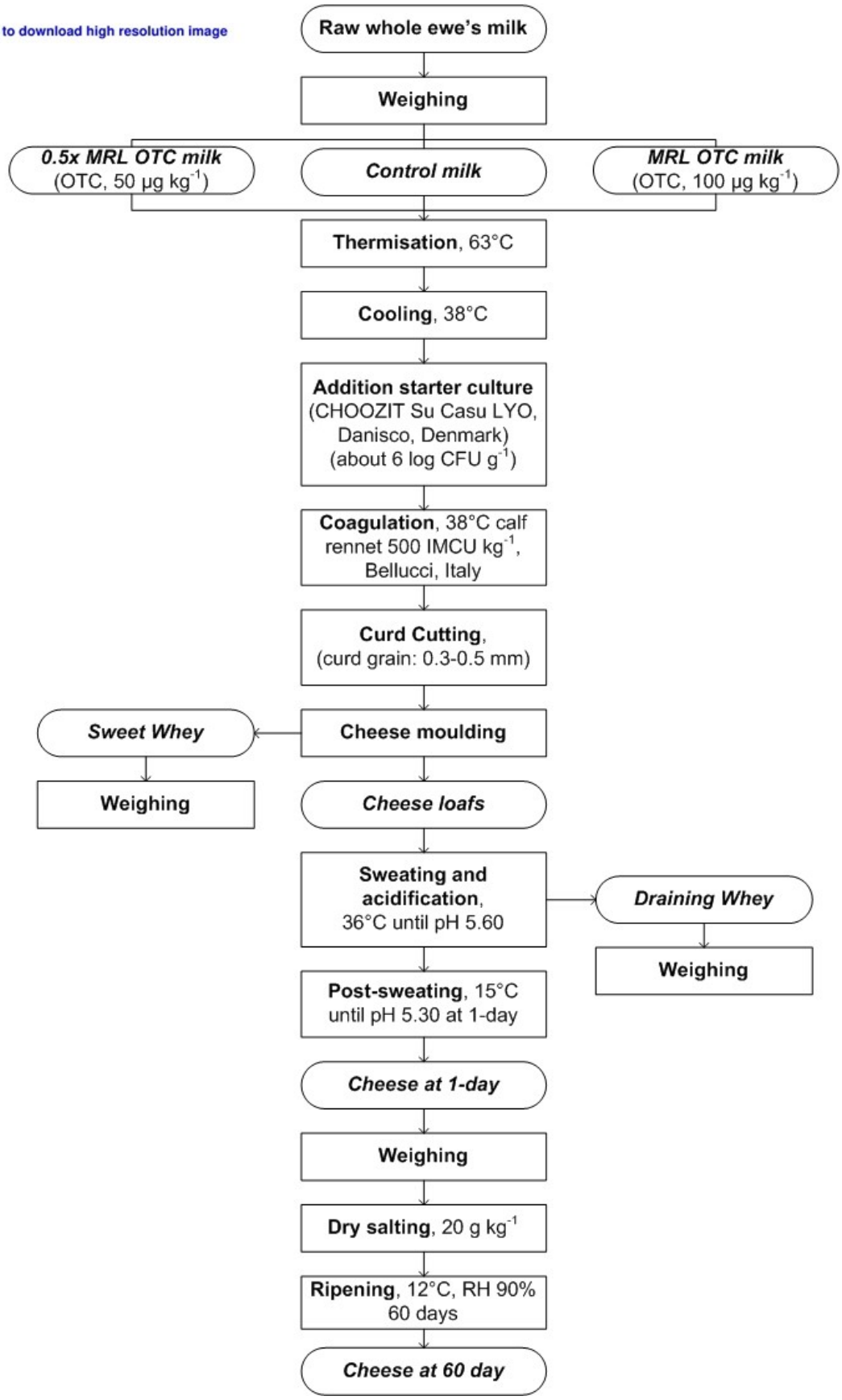

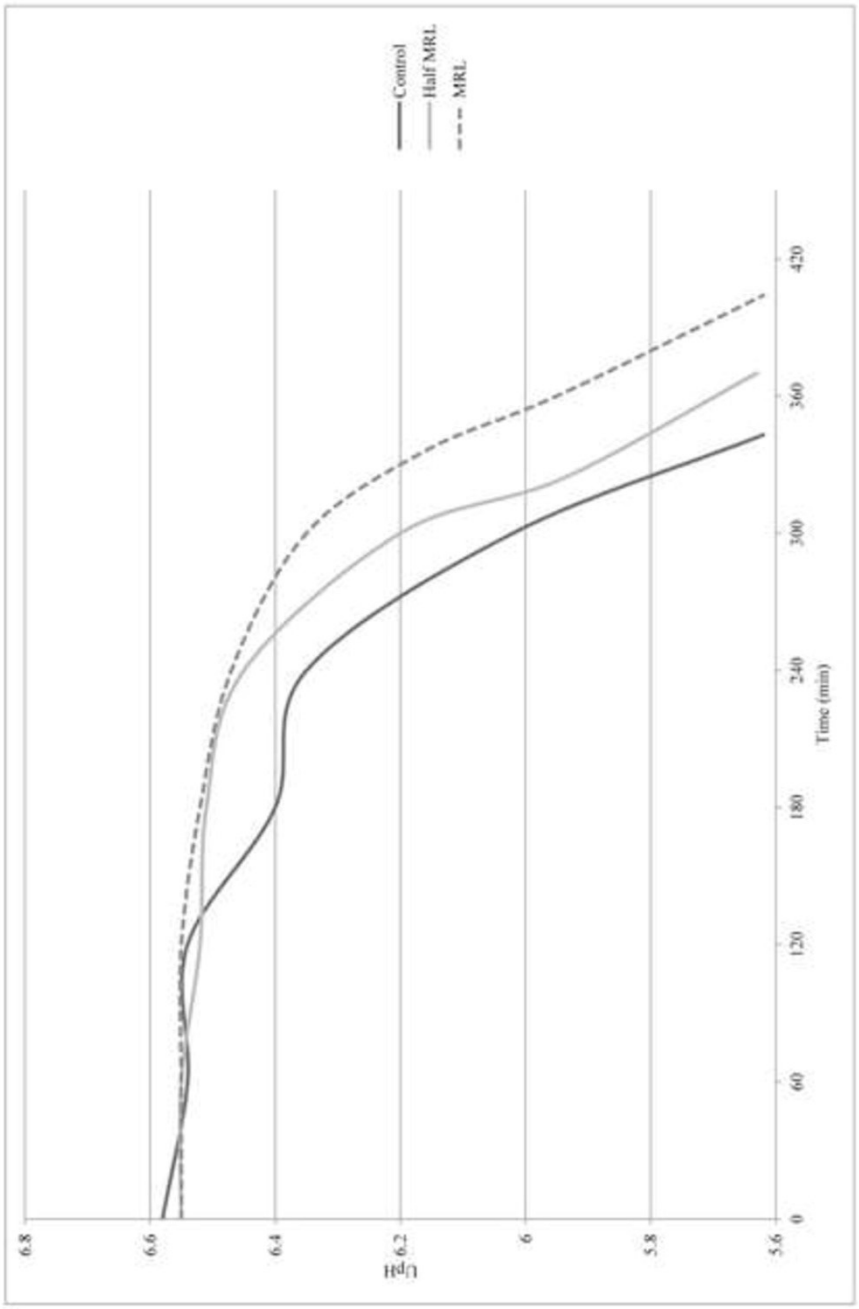

Figure 3
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Figure captions

Fig. 1. Heat penetration curve of the adopted thermal treatment.

Fig. 2. Cheese-making process.

Fig. 3. Acidification curves.

7 Chapter IV: Monitoring of early acidification phase and culture starter development in cheese produced from thermised ovine milk spiked with OTC at MRL level

1. Introduction

Considering the results discussed in chapter III, a new experiment was set-up to investigate the effect of antibiotic residues on the development of the starter culture, previously adopted during the early acidification phase. Indeed, based on the observed correlation between the dose of OTC spiked in sheep milk and the delay recorded during the acidification phase, the development of the starter culture was monitored during the first 6 hours of cheese acidification. In fact, in the experiment reported in chapter III, the observed delay in acidification of thermised spiked milk did not affect starter lactic acid bacteria (SLAB) viable counts in 1-day cheese, leading to hypothesize a temporary OTC effect, limited on the early acidification phase. CHOOZIT® *Su Casu* starter culture employed in semi-cooked and cooked cheese production, authorized for Pecorino Romano and Pecorino Sardo PDO cheeses, was used in all the pilot plant experimental cheese-making. It is a thermophilic starter culture employed in cheese-making to produce semi- and cooked cheese, authorized for the production of Pecorino Romano PDO and Pecorino Sardo PDO. CHOOZIT® *Su Casu* is a thermophilic starter culture with a fast acidification rate, composed of *Streptococcus thermophilus, Lactobacillus lactis* and *Lactobacillus helveticus.* In order to better understand the role of OTC on starter culture development, the trial was conducted with the same cheese-making process adopted for the trials reported in chapter III was followed, focusing the investigation on OTC at MRL level (100 $\mu g\ kg^{-1}$).

2. Materials and Methods

2.1 Chemical and reagents

Oxytetracycline hydrochloride (OTC, purity ≥96.7%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of OTC was prepared by diluting the pure standard in methanol to a final concentration of 1 g L^{-1} and stored in the dark at -20 °C until their use for the analytical procedure.

2.2 Cheese-making process

Ovine milk was collected from an experimental flock of Sarda breed sheep at AGRIS Sardegna Research Agency (Olmedo, Sassari, Italy). Sheep were in good health and did not undergo antibiotic treatments. The mean chemical composition of the milk was as follows: pH 6.68 ± 0.01; protein 4.98 ± 0.32% (w/w); fat, 5.56 ± 0.11% (w/w); casein, 3.85 ± 0.27% (w/w); and lactose 4.72 ± 0.07% (w/w). The milk was divided into 2 vats containing 36 kg each. The first one was the control (not spiked) and the second one was spiked with OTC at MRL level (100 µg kg^{-1}). Subsequently, raw milk was heated up to 63°C in each vat and immediately cooled until 38°C.

Then, the starter culture was inoculated (6 log CFU g^{-1}, CHOOZIT® *Su Casu* LYO, Danisco, Denmark) and calf rennet added (500 IMCU kg^{-1}, Bellucci, Italy), following the cheese-making process described in figure 7, in order to obtain uncooked, hard, pecorino cheeses ripened until 60 days. From each vat, 6 cheeses were obtained and sampled at different time for the microbiological analysis. The

experiment was replicated three times in a short period (3 consecutive weeks, in February 2016) to minimize the possible effect of milk composition.

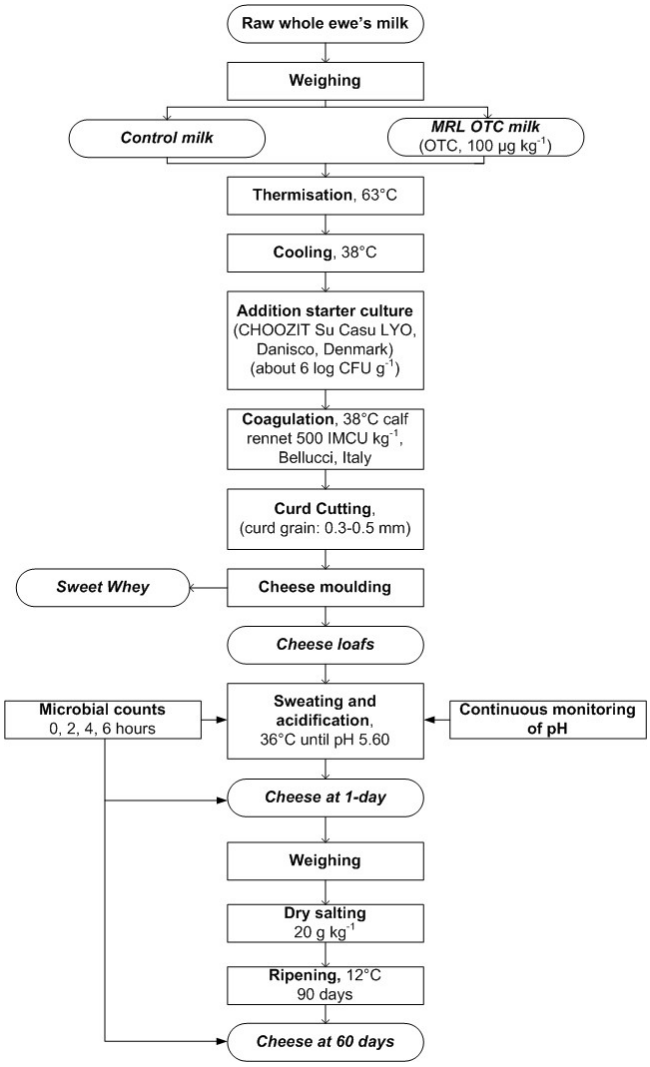

Fig. 7 Cheese-making process

2.3 Milk composition

The parameters of milk composition were evaluated by using MilkoScan FT+ (Foss, Hillerød, Denmark) as described in ISO 9622 (ISO, 2013).

The evolution of pH during the acidification phase of cheese (until pH 5.6) was monitored in continuous (pH-meter Liquiline CM448, Endress+Hauser coupled with CERAGEL CPS71D).

2.4 Microbiological analyses

Samples of curd at 0, 2, 4 and 6 hours from starter inoculum (early acidification phase), 1 and 60 days ripened cheeses were prepared according to the IDF standard 122C (1996). Viable counts were carried out to enumerate: starter lactic acid bacteria (SLAB), i.e. thermophilic cocci and lactobacilli, on M17 agar and MRS agar media (Microbiol, Cagliari, Italy), respectively, incubated at 45 °C, in anaerobiosis for 72 h; non-starter lactic acid bacteria (NSLAB), i.e. mesophilic lactobacilli, on FH agar medium (Isolini et al., 1990), incubated at 37 °C, in anaerobiosis for 72 h, sampled at 0 hour, 1 and 60 days cheese; Enterococci on KAA medium (Microbiol), incubated at 42 °C, in aerobiosis, for 18-24 h; coliform bacteria, on VRBA MUG (Microbiol), incubated at 37 °C, in aerobiosis for 18-24 h.

2.5 Statistical analysis

Statistical analysis was performed by Statgraphics Centurion XVI for Windows software package (version 16.2.04; Statpoint Technologies, Inc. Warrenton, Virginia, VA, USA). Analysis of variance (ANOVA) was carried out using the general linear model (GLM) to determine the effects of treatment and replicate on each response variable. Tukey's multiple comparison test was used for paired comparison of treatment means ($P < 0.05$).

3. Results and discussion

The delay to reach pH 5.60 observed for OTC spiked milk at MRL level in comparison with control samples, during the early acidification phase was about 80 minutes, as already noticed in the experiment reported in chapter III (Fig. 8).

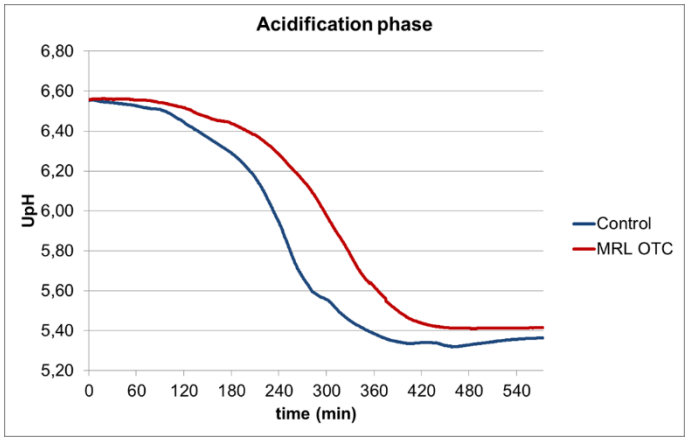

Fig. 8 Profile of acidification phase of cheeses

No differences were recorded in the viable counts of thermised milk, before and after the inoculum of the starter culture (Table 7). Despite the delay observed, no differences were observed in SLAB (Table 8), NSLAB and Enterococci (Table 9), during the first 6 hours and in the cheeses.

Relevant variations were observed on the development of coliform bacteria in MRL level samples. In fact, significant differences ($P<0.05$) in coliform bacteria counts, between control and experimental samples, in 6-hour and 1-day cheeses, were found (Table 10), confirming the correlation between the presence of residues at MRL level and the acidification delay that allowed coliform bacteria to reach a higher concentration in OTC spiked samples. The difference in coliform bacteria counts between control and experiment disappeared at the end of ripening period.

Table 7
Thermised milk and thermised milk + starter viable counts [a].

Parameter	Thermised milk						Thermised milk + starter					
	Control			OTC MRL			Control			OTC MRL		
Thermophilic cocci	1.9	±	0.1	2.5	±	0.8	5.9	±	0.1	5.8	±	0.1
Thermophilic lactobacilli	2.1	±	0.4	2.0	±	0.5	4.3	±	0.1	4.5	±	0.1
Mesophilic lactobacilli	1.5	±	0.3	1.4	±	0.2	1.4	±	0.3	1.4	±	0.3
Enterococci	0.7	±	0.6	1.1	±	0.7	0.4	±	0.8	0.6	±	1.0
Coliform	0	±	0	0	±	0	0	±	0	0	±	0

[a] Values log CFU mL^{-1} are means ± standard deviation (n=3). Values within a row not sharing a common superscript letters were significantly different ($P < 0.05$).

Table 8
Early acidification phase, 1 and 60 days cheese SLAB viable counts[a].

Time	Thermophilic cocci						Thermophilic lactobacilli					
	Control			OTC MRL			Control			OTC MRL		
0 h	6.5	±	0.1	6.3	±	0.1	4.9	±	0.1	4.9	±	0.1
2 h	7.2	±	0.1	7.4	±	0.3	4.8	±	0.1	4.7	±	0.3
4 h	7.7	±	0.1	7.7	±	0.6	4.8	±	0.3	4.8	±	0.2
6 h	9.0	±	0.2	8.8	±	0.2	4.9	±	0.3	5.1	±	0.3
1 day	8.4	±	0.7	8.9	±	0.6	6.0	±	0.2	5.8	±	0.3
60 day	8.2	±	0.6	8.8	±	0.4	5.0	±	0.1	5.2	±	0.3

[a] Values log CFU g^{-1} are means ± standard deviation (n=3). Values within a row not sharing a common superscript letters were significantly different ($P < 0.05$).

Table 9
Early acidification phase, 1 and 60 days cheese NSLAB viable counts[a].

Time	Mesophilic lactobacilli						Enterococci					
	Control			OTC MRL			Control			OTC MRL		
0 h	2.0	±	0.2	1.8	±	0.5	1.5	±	0.3	1.7	±	0.8
2 h	n.d.			n.d.			1.7	±	0.3	1.9	±	0.9
4 h	n.d.			n.d.			2.0	±	0.3	2.2	±	1.2
6 h	n.d.			n.d.			2.3	±	0.2	2.4	±	1.1
1 day	2.2	±	0.2	2.0	±	0.3	3.1	±	0.4	3.1	±	0.9
60 day	8.1	±	0.7	8.4	±	0.3	4.6	±	0.2	4.4	±	0.2

[a] Values log CFU g^{-1} are means ± standard deviation (n=3). Values within a row not sharing a common superscript letters were significantly different ($P < 0.05$).
n.d. = not determined, sampled at 0 hour, 1 and 60-day cheese.

Table 10
Early acidification phase, 1 and 60 days cheese coliform bacteria viable counts[a].

Time	Coliform bacteria					
	Control			OTC MRL		
0 h	0.8	±	0.8	0.9	±	0.8
2 h	1.5	-	0.7	1.8	-	0.9
4 h	2.4	-	2.0	3.2	-	0.7
6 h	2.7	-	0.7 [b]	3.9	-	0.7 [a]
1 day	3.1	±	0.4 [b]	4.6	±	0.4 [a]
60 day	0.7	±	1.2	1.5	±	1.3

[a] Values log CFU g^{-1} are means ± standard deviation (n=3). Values within a row not sharing a common superscript letters were significantly different ($P < 0.05$).

4. Conclusion

The delay in acidification, observed in cheese produced from spiked milk at MRL level, allowed the development of coliform bacteria at higher level compared to the control. This aspect may have negative technological implications during the cheese-making causing defects in cheese and constitute a risk for human health, linked to the consumption of short-ripened cheese.

A temporary SLAB lactose metabolism/lactic acid production ability inhibition by oxytetracycline was hypothesized. However, further investigations focused on the study of singular bacterial strains content in the starter culture would be needed to understand the reason why OTC spiked samples starter culture viable counts do not significantly differ from the control ones, even though a delay in acidification occurred when residues of OTC were present in cheese.

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9 General conclusions

The Ph.D. project contributed to increase the knowledge on the fate and the technological effects of the presence of low-level residues of oxytetracycline in ovine milk.

In the first work, the applicability of a microscale cheese-making system to predict the partition of oxytetracycline from spiked ovine milk and obtained fractions (cheese, whey) was evaluated. The adopted screening system highlighted a concentration effect of the molecule in the curd at the end of the cheese-making process. The system, also being characterized by a good repeatability (4%), was unable to explain the whole distribution of the molecule, probably due to the small volumes of processed milk, and did not recover a high quote of the molecule.

The second work permitted to calculate, with an innovative approach, the distribution of the molecule between whey and cheese by performing experiments in a pilot plant on OTC spiked raw ovine milk at MRL level by the application of a conventional cheese-making process. Cheese produced from milk spiked with OTC at MRL level showed a significant delay in acidification, close to 60 minutes, before reaching pH 5.60 during manufacture.

Results indicated that most of the OTC (about 60%) added to the whole raw ovine milk was recovered in the 1-day-old cheese. Moreover, a reduction of the residual concentration of OTC with the ripening was observed, suggesting its partial degradation, but no epimers were detected by LC-MS/MS.

A subsequent study by Shappel et al. (2017), based on microscale cheese-making, provided a positive correlation between the lipophilicity and the distribution of

OTC among curd (14%) and whey, thus reporting a distribution of the molecule that appears in contrast with those here exposed. However, some experimental differences contribute to make the two studies hardly comparable, such as: 1) the chemical composition of ovine milk and the physical-chemical characteristics of its constituents 2) the adopted cheese-making technology and the scale of experiments 3) the analytical method adopted in OTC measurements. Studies which aim to verify the distribution of antibiotics from milk to cheese, should take into consideration the type of milk used, the cheese-making technology and be performed in realistic conditions. In fact, the composition of the curd and its interaction with constituents in vat depends on milk composition and on many variables related to the whole adopted technology, as discussed in section 1.3 and 1.4.

The third study was set-up considering that most ovine milk produced in Sardinia is thermised before cheese-making. The effect of thermal treatment on partition of OTC between cheese and whey was studied.

Milk thermisation did not affect OTC recovery, partition and cheese chemical composition, probably because of the protective effect of the higher total solid content of the ovine milk, especially fat. Residues in milk caused a dose-dependent delay to reach pH 5.60, which was significant at MRL level (406 ± 2 min) compared to half MRL level and the control (363 ± 30 and 328 ± 24 min, respectively). Cheese was able to retain a great amount of OTC (about 80%). The overall molecule recovered, close to 100%, obtained in this study could be ascribable to the better performances provided by the adopted analytical method,

involving LC-HRMS. The OTC residues in cheese underwent a modest decrease during ripening, but no epimers were detected by LC-HRMS.

Microflora development in the presence of OTC residues was monitored by viable counts. Despite the delay during the acidification phase in both levels, no significant differences were found in 1-day cheese starter counts. The delay permitted a growth of coliform bacteria able to reach 6 log CFU g^{-1}, with potential negative technological and safety implications in short-ripening cheeses.

During the fourth study, the early acidification phase was monitored (between 0 and 6 hours from the starter culture inoculation) in order to study the effect of oxytetracycline residues on the development of the starter culture.

As previously observed, the cheeses obtained from MRL spiked milk reached pH 5.60 80 minutes later than the control ones, but no significant differences in the viable counts of SLAB during the first 6 hours and in the cheeses were observed. This evidence lead to suppose the dearth of difference in viable counts of SLAB being ascribable to a temporary inhibition of lactose metabolism/lactic acid production, due to the presence of oxytetracycline.

However, the delay in acidification caused by the presence of oxytetracycline residues allowed coliform bacteria to develop at a significant ($P < 0.05$) higher level in the spiked samples compared to the control ones.

In conclusion, under our conditions, the oxytetracycline added in ovine milk was mainly recovered in the cheese causing different consequences. Milk thermisation did not reduce OTC concentration both at half and at MRL levels. In the absence of reduction, problems can be caused for consumers during the consumption of

fresh cheese. Oxytetracycline is only partially degraded at the end of the ripening period, causing a risk not negligible through the consumption of ripened cheese. Additionally, residues in milk caused a dose-dependent delay to reach prefixed pH (5.60), without showing a difference on the growth of starter culture between control and experiment levels. The delay in acidification caused a higher development of coliform bacteria in the presence of OTC, which constitutes a hazard for human health, especially in short-ripening cheese.

The outcomes of the present Ph.D. project could be helpful to fill the legislative gap about the limits of oxytetracycline residues in milk-derived products.

In order to support official decisions to set possible limits in cheese, studies focused on the distribution of antimicrobials in dairy products should be performed taken into account the class of antibiotics, the milking species, cheese-making technology, chemico-physical parameters (for instance fat, proteins and moisture) and ripening time of cheese.

10 Other works

Oral and poster presentations

10.1. Oral presentations

The fate of oxytetracycline from spiked ovine milk to cheese

Cabizza. R.

XXII Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology. 20th-22th September 2017. Bolzano. Italy

22nd Workshop on the *Developments in the Italian PhD Research on Food Science Technology and Biotechnology,* Free University of Bozen, Bozen, September 20th-22nd, 2017

The fate of oxytetracycline from spiked ovine milk to cheese

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Tutor: Dr. Pietro Paolo Urgeghe;
Co-Tutor: Dr. Roberta Comunian (AGRIS Sardegna); Co-Tutor: Dr. Cecilia Testa (IZS Sardegna)

The Commission Regulation (EU) 37/2010 states the Maximum Residue Limit MRL on pharmacologically active substances in foodstuffs of animal origin, included milk, but no limits are fixed for derived-milk products. The aim of this PhD thesis project is to study the distribution, between cheese and whey, of oxytetracycline antibiotic added in ovine milk, and to verify possible negative technological effects throughout the cheese-making process.

Studio degli equilibri di ripartizione degli antibiotici veterinari nei derivati del latte

Il Regolamento EU 37/2010 stabilisce il limite massimo di residuo (MRL) per le sostanze farmacologicamente attive negli alimenti di origine animale, tra cui il latte, ma allo stato attuale non sono fissati valori di riferimento per formaggio e altri prodotti derivati. L'obiettivo generale del progetto di dottorato è studiare la ripartizione dell'antibiotico ossitetraciclina, aggiunto in latte ovino, tra le componenti che derivano dal processo di trasformazione casearia (siero e formaggio), e verificare se tali livelli di residuo nel latte, possano influenzare negativamente gli aspetti tecnologici del processo e avere un effetto sui prodotti ottenuti.

Key words: Ovine milk, antibiotic, cheese, partition, MRL, oxytetracycline

Performance of Eclipse Farm test coupled with e-Reader for screening of antibiotics in sheep and goat milk

Cabizza. R.

Joint Seminar of the FAO-CIHEAM Network on Sheep and Goats.

3rd-5th October 2017. Vitoria. Spain

Title	**Performance of Eclipse Farm test coupled with e-Reader for screening of antibiotics in sheep and goat milk**
Authors	J. GIRALDO (1), R. CABIZZA (2), D. SANZ (3), L. MATA (3), M. P. MOLINA (1) (1)Universitat Politecnica de Valencia, Institute of Animal Science and Technology. . Camino de Vera , 14, 46022, Valencia, SPAIN. (2)Universitá degli Studi di Sassari, Dipartimento di Agraria. Via De Nicola, 9, 07100, Sassari, ITALY. (3)ZEULAB s.L., R&D Department. Bari, 25 dplo, 50197, Zaragoza, SPAIN.
Keywords	Antimicrobial residues, screening test, sheep milk, goat milk

The presence of antibiotic residues in milk is a concern because of technological and analytical reasons but mostly due to their side effects in human health. Although many methods have been developed to analyse antibiotic residues in cow milk, such methods not always correctly work with sheep and goat milk. In this work we present a study of the performance of a new system for the screening of antimicrobial residues in sheep and goat milk. The method combines a microbial inhibitor test (Eclipse Farm) and a device (e-Reader) that integrates incubation at 65ºC and continuous monitoring of the colour change. Thus, it determines automatically the end-point of the assay and interprets results in an objective way. A preliminary study demonstrated the necessity to include a one-hour diffusion period at room temperature. The performance of the new system was validated according to the European Commission Decision 2002/657/EC. Sensitivity of the new system was evaluated on 12 molecules from several families of antimicrobials. The detection limits were close to the European maximum residue limits (MRL). Detection capabilities (CCβ) were also determined for 6 molecules representing the main antimicrobial groups used in dairy husbandry (penicillins, cephalosporins, tetracyclins, sulphonamides, macrolides and aminoglycosides). All molecules were detected at the MRL level. Robustness was also studied, demonstrating that the new method was unaffected by reasonable changes in the procedure. Eclipse Farm coupled to e-Reader has proved to be a valuable tool for screening a broad-spectrum of antimicrobial residues in sheep and goat milk.

Validation of a microbial inhibition test based on Eclipse Farm coupled with e-Reader for screening β-lactam and tetracycline antibiotics in goat's cheese whey

Cabizza. R.

Joint Seminar of the FAO-CIHEAM Network on Sheep and Goats.

3rd-5th October 2017. Vitoria. Spain

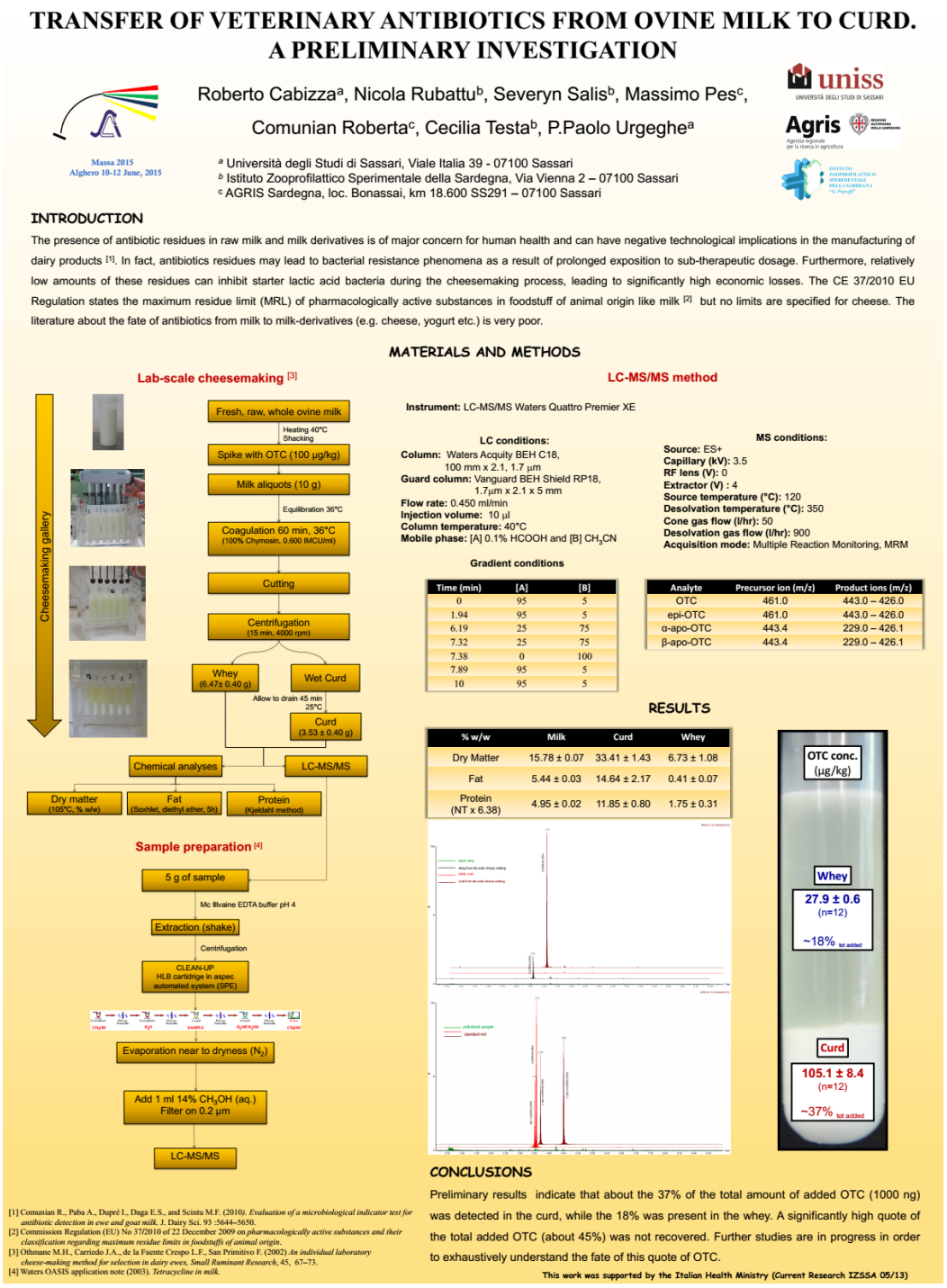
Title	**Validation of a microbial inhibition test based on Eclipse Farm coupled with e-Reader for screening β-lactam and tetracycline antibiotics in goat's cheese whey**
Authors	J. GIRALDO (1), R. CABIZZA (2), L. MATA (3), M. P. MOLINA (1) (1)Universitat Politecnica de Valencia, Institute of Animal Science and Technology, Camino de Vera, 14, 46022, Valencia, SPAIN. (2)Università degli Studi di Sassari, Dipartimento di Agraria. Via De Nicola , 9, 07100, Sassari, ITALY. (3)ZEULAB, S.L. , R&D Department. Bari, 25 dplo, 50197, Zaragoza, SPAIN.
Keywords	Antibiotic residues, screening test, whey

The presence of antimicrobial residues in milk and dairy products, such as cheese, could cause negative technological effects and represents a risk for consumer health. In the cheese-making process, antibiotics could be retained in curd or eliminated in whey to a greater or lesser extent. Whey is a by-product used in the manufacture of foodstuffs for human consumption, animal feeding, among others. In order to guarantee food safety and animal health, it would be convenient to establish an analytical strategy to screen antibiotics in whey. Thus, a new system for screening antibiotics in raw milk was developed, coupling a microbial inhibitor tube test (Eclipse Farm) and a device (e-Reader) based on incubation and color change continuous monitoring. The aim of this work was to study the performance of the Eclipse Farm test coupled with the e-Reader for the detection of β-lactams and tetracyclines in goat's cheese whey. A preliminary study demonstrated the necessity to include a one-hour diffusion period at room temperature and to adjust pH in the analysis of acid whey samples for a correct interpretation of the results. The performance was validated in agreement with European Commission Decision 2002/657/EC. Specificity was evaluated analyzing one hundred whey samples presenting very high values. Detection limits for amoxicillin, cephalexin and oxytetracycline in fortified goat whey were calculated, and the detection capabilities (CCβ) were at or below the MRL levels. In conclusion, Eclipse Farm coupled to e-Reader represents an appropriate method to screen β-lactams and tetracycline residues in whey.

Transfer of veterinary antibiotics from ovine milk to curd. A preliminary investigation

Cabizza. R.

Massa 2015. 10th-12th June 2015. Alghero (SS). Italy

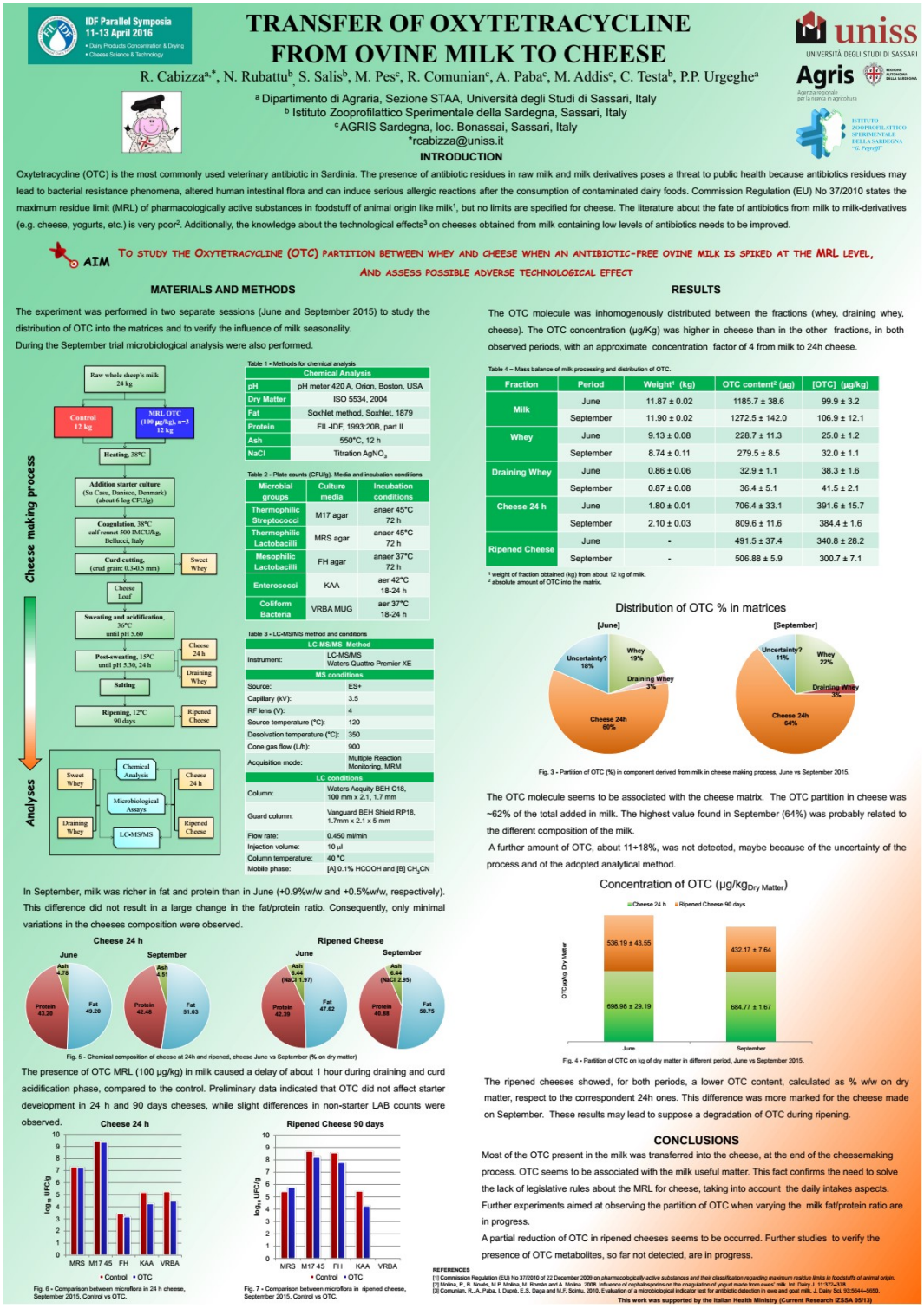


Roberto Cabizza, *Oxytetracycline residues from spiked ovine milk to cheese: technological implications* Tesi di dottorato in Scienze Agrarie – *Curriculum* "Biotecnologie Microbiche Agroalimentari". Ciclo XXX Università degli Studi di Sassari.
Anno Accademico 2016/2017

Transfer of oxytetracycline from milk to cheese

Cabizza. R.

IDF Parallel Symposia. 11th -13th April 2016. Dublin. Ireland



IDF Parallel Symposia 11-13 April 2016 • Dairy Products Concentration & Drying • Cheese Science & Technology

TRANSFER OF OXYTETRACYCLINE FROM OVINE MILK TO CHEESE

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INTRODUCTION

Oxytetracycline (OTC) is the most commonly used veterinary antibiotic in Sardinia. The presence of antibiotic residues in raw milk and milk derivatives poses a threat to public health because antibiotics residues may lead to bacterial resistance phenomena, altered human intestinal flora and can induce serious allergic reactions after the consumption of contaminated dairy foods. Commission Regulation (EU) No 37/2010 states the maximum residue limit (MRL) of pharmacologically active substances in foodstuff of animal origin like milk[1], but no limits are specified for cheese. The literature about the fate of antibiotics from milk to milk-derivatives (e.g. cheese, yogurts, etc.) is very poor[2]. Additionally, the knowledge about the technological effects[3] on cheeses obtained from milk containing low levels of antibiotics needs to be improved.

AIM TO STUDY THE OXYTETRACYCLINE (OTC) PARTITION BETWEEN WHEY AND CHEESE WHEN AN ANTIBIOTIC-FREE OVINE MILK IS SPIKED AT THE MRL LEVEL, AND ASSESS POSSIBLE ADVERSE TECHNOLOGICAL EFFECT

MATERIALS AND METHODS

The experiment was performed in two separate sessions (June and September 2015) to study the distribution of OTC into the matrices and to verify the influence of milk seasonality.
During the September trial microbiological analysis were also performed.

Table 1 - Methods for chemical analysis

Chemical Analysis	
pH	pH meter 420 A, Orion, Boston, USA
Dry Matter	ISO 5534, 2004
Fat	Soxhlet method, Soxhlet, 1879
Protein	FIL-IDF, 1993:20B, part II
Ash	550°C, 12 h
NaCl	Titration $AgNO_3$

Table 2 - Plate counts (CFU/g). Media and incubation conditions

Microbial groups	Culture media	Incubation conditions
Thermophilic Streptococci	M17 agar	anaer 45°C 72 h
Thermophilic Lactobacilli	MRS agar	anaer 45°C 72 h
Mesophilic Lactobacilli	FH agar	anaer 37°C 72 h
Enterococci	KAA	aer 42°C 18-24 h
Coliform Bacteria	VRBA MUG	aer 37°C 18-24 h

Table 3 - LC-MS/MS method and conditions

LC-MS/MS Method	
Instrument:	LC-MS/MS Waters Quattro Premier XE
MS conditions	
Source:	ES+
Capillary (kV):	3.5
RF lens (V):	4
Source temperature (°C):	120
Desolvation temperature (°C):	350
Cone gas flow (L/h):	900
Acquisition mode:	Multiple Reaction Monitoring, MRM
LC conditions	
Column:	Waters Acquity BEH C18, 100 mm x 2.1, 1.7 mm
Guard column:	Vanguard BEH Shield RP18, 1.7mm x 2.1 x 5 mm
Flow rate:	0.450 ml/min
Injection volume:	10 µl
Column temperature:	40 °C
Mobile phase:	[A] 0.1% HCOOH and [B] CH_3CN

In September, milk was richer in fat and protein than in June (+0.9%w/w and +0.5%w/w, respectively). This difference did not result in a large change in the fat/protein ratio. Consequently, only minimal variations in the cheeses composition were observed.

Fig. 5 - Chemical composition of cheese at 24h and ripened, cheese June vs September (% on dry matter)

The presence of OTC MRL (100 µg/kg) in milk caused a delay of about 1 hour during draining and curd acidification phase, compared to the control. Preliminary data indicated that OTC did not affect starter development in 24 h and 90 days cheeses, while slight differences in non-starter LAB counts were observed.

Fig. 6 - Comparison between microflora in 24 h cheese, September 2015, Control vs OTC.

Fig. 7 - Comparison between microflora in ripened cheese, September 2015, Control vs OTC.

RESULTS

The OTC molecule was inhomogenously distributed between the fractions (whey, draining whey, cheese). The OTC concentration (µg/Kg) was higher in cheese than in the other fractions, in both observed periods, with an approximate concentration factor of 4 from milk to 24h cheese.

Table 4 – Mass balance of milk processing and distribution of OTC.

Fraction	Period	Weight[1] (kg)	OTC content[2] (µg)	[OTC] (µg/kg)
Milk	June	11.87 ± 0.02	1185.7 ± 38.6	99.9 ± 3.2
	September	11.90 ± 0.02	1272.5 ± 142.0	106.9 ± 12.1
Whey	June	9.13 ± 0.08	228.7 ± 11.3	25.0 ± 1.2
	September	8.74 ± 0.11	279.5 ± 8.5	32.0 ± 1.1
Draining Whey	June	0.86 ± 0.06	32.9 ± 1.1	38.3 ± 1.6
	September	0.87 ± 0.08	36.4 ± 5.1	41.5 ± 2.1
Cheese 24 h	June	1.80 ± 0.01	706.4 ± 33.1	391.6 ± 15.7
	September	2.10 ± 0.03	809.6 ± 11.6	384.4 ± 1.6
Ripened Cheese	June	-	491.5 ± 37.4	340.8 ± 28.2
	September	-	506.88 ± 5.9	300.7 ± 7.1

[1] weight of fraction obtained (kg) from about 12 kg of milk.
[2] absolute amount of OTC into the matrix.

Distribution of OTC % in matrices

Fig. 3 - Partition of OTC (%) in component derived from milk in cheese making process, June vs September 2015.

The OTC molecule seems to be associated with the cheese matrix. The OTC partition in cheese was ~62% of the total added in milk. The highest value found in September (64%) was probably related to the different composition of the milk.
A further amount of OTC, about 11÷18%, was not detected, maybe because of the uncertainty of the process and of the adopted analytical method.

Concentration of OTC (µg/$kg_{Dry\ Matter}$)

Fig. 4 - Partition of OTC on kg of dry matter in different period, June vs September 2015.

The ripened cheeses showed, for both periods, a lower OTC content, calculated as % w/w on dry matter, respect to the correspondent 24h ones. This difference was more marked for the cheese made on September. These results may lead to suppose a degradation of OTC during ripening.

CONCLUSIONS

Most of the OTC present in the milk was transferred into the cheese, at the end of the cheesemaking process. OTC seems to be associated with the milk useful matter. This fact confirms the need to solve the lack of legislative rules about the MRL for cheese, taking into account the daily intakes aspects. Further experiments aimed at observing the partition of OTC when varying the milk fat/protein ratio are in progress.
A partial reduction of OTC in ripened cheeses seems to be occurred. Further studies to verify the presence of OTC metabolites, so far not detected, are in progress.

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Roberto Cabizza, *Oxytetracycline residues from spiked ovine milk to cheese: technological implications*
Tesi di dottorato in Scienze Agrarie – *Curriculum* "Biotecnologie Microbiche Agroalimentari". Ciclo XXX
Università degli Studi di Sassari.
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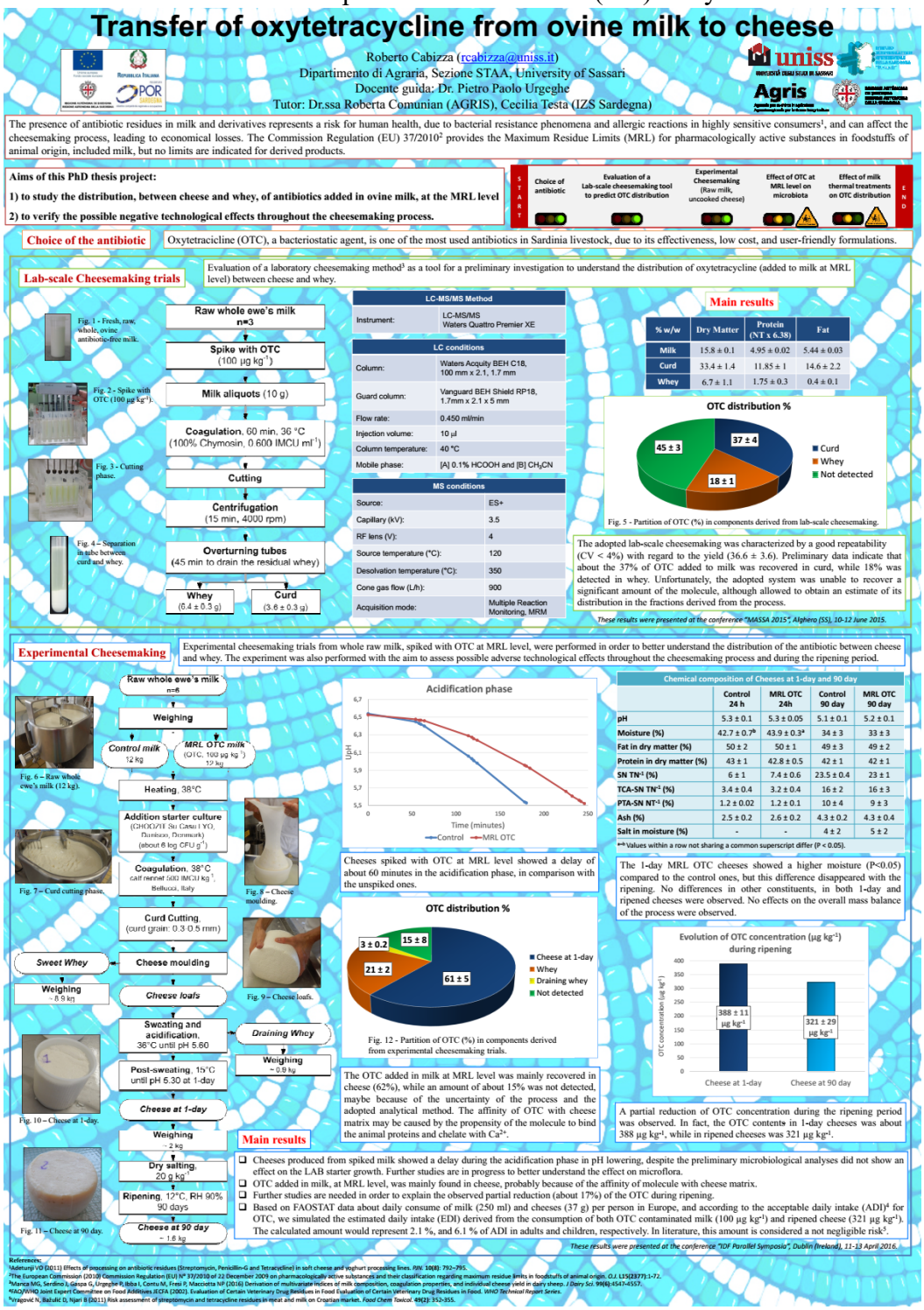
Transfer of oxytetracycline from milk to cheese

Cabizza. R.

XXI Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology.

14th -16th September 2016. Portici (NA). Italy



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The fate of oxytetracycline in spiked sheep's milk during cheese-making and cheese ripening

Cabizza. R.

Networking: tool for an excellent research. 6th April 2017. Roma. Italy

Networking: strumento per una ricerca di eccellenza

The fate of oxytetracycline in spiked sheep's milk during cheese-making and cheese ripening

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This paper presents the results of a study on the effects and the distribution of oxytetracycline (OTC), added to ovine milk at MRL concentration, during cheese-making and cheese ripening.
The evidences currently available concern partition of antiparasitic drugs from milk to milk-derivatives and only a few studies investigated the technological effects related to low doses of antibiotics on cheese-making processes.
The OTC distribution was assessed through the cheese-making process starting from both raw and thermized milk.
OTC caused a dose dependent delay in pH lowering of the curd. Despite that, no effects on the total microbial count or on cheese composition and ripening were observed.
Similarly, MRL OTC did not influence mass balance or the recovery of fat and protein in cheese.
OTC added to raw whole ovine milk was mainly recovered in the 1-day-old cheese (60% and 80% in cheese from raw and thermized milk, respectively).
The absolute OTC amount decreased in the cheese during ripening, leading to suppose the contribution of degradation phenomena.

The authors report published data and partial data that will be available for potential future publications.

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