UNIVERSITY OF NAPLES "FEDERICO II"

DEPARTMENT OF PHARMACY

Ph.D. THESIS

IN

"PHARMACEUTICAL SCIENCE"

Study of milk contaminants and re-evaluation of whey: potential use of dairy by-product in functional food

Supervisor: **Coordinator:**
Ch.mo Prof. Alberto Ritieni **Prof.ssa Maria Valeria D'Auria**

Candidate:
Dr. Luana Izzo

XXXI cycle
Academic year 2015/2018

PREFACE

This Ph.D. thesis is submitted as a requirement for obtaining the Ph.D. Degree at the University of Naples Federico II (Italy). It is written based on three-years research conducted by the author, Luana Izzo, both at the Department of Pharmacy in Naples, under the supervision of Prof. Alberto Ritieni, and at the Department of Food Chemistry and Toxicology in Valencia (Spain) under the supervision of Prof. Giuseppe Meca.

Luana Izzo

Naples, October 2018

Niente nella vita va temuto, dev'essere solamente compreso. Ora è tempo di comprendere di più, cosi possiamo temere di meno.

Marie Curie

La scienza come la vita è imperfetta perchè ogni volta che risolve un problema, ne crea almeno dieci nuovi.

George Bernard Shaw

Alcuni amici non saranno mai distanti,

come le stelle, non puoi vederle sempre ma sai che sono lí!

Gràcies per tot Yelko!

ABSTRACT

Milk is the most widely consumed beverage in the world, not counting water, which provides high-quality nutrition to infants, children, and adults, due to its high content of micro- and macronutrients. Despite the benefits that milk provides from a nutritional point of view, it may also be a vehicle of food contaminants, such as mycotoxins and veterinary drug residues (VDs), due to nasty agricultural practices or improper usage of these drugs. In this sense, the first part of my project includes the studies regarding the topics mentioned above.

Hence, a multi-residue method based on a QuEChERS extraction for the simultaneous determination of veterinary drug residues *(n=61)* and mycotoxins *(n=46)* in milk was developed by using ultra high performance liquid chromatography coupled with high resolution mass spectrometry (UHPLC Q-Exactive Orbitrap HRMS). The recoveries were in a range of 72-93% at three spiking levels, with repeatability and reproducibility results expressed as relative standard deviations (RSDs) lower than 7% and 14%, respectively. The limits of quantification (LOQs) were in the range of 0.01-50 pg/mL. Matrix effects obtained were in the range of 75-97%. The results obtained from the study showed that none of the analyzed samples *(n=56)* were contaminated with mycotoxins, whereas the presence of up to seven veterinary drug residues in 50% of analyzed samples was detected (range of 0.007-4.53 ng/mL). None of the analyzed VDs had a concentration level higher than their permitted limits, except for benzylpenicillin procain (4.53 ng/mL). In the post-target screening, 53 contaminants were tentatively identified. Regarding the incidence of mycotoxins, AFM2 contamination was displayed in >70% of analyzed samples. A significant percentage (>80%) has been shown by betamethasone, prednisolone and oxfendazole. Special focus

should be paid to the latter, considering that the maximum residue limit permitted in EC 37/2010 is really low, 0.3; 6; 10 μg/Kg, respectively. In addition, other eleven VDs identified in analyzed milk samples are prohibited in milk by the regulation in force (EC 37/2010). From the obtained values seems to be clear the necessity of a continuous monitoring of contaminants in the milk production chain.

The second part of my Ph.D. project was based on the re-evaluation of whey, one of the highest polluting liquid wastes of the dairy industry. Environmental and economic problems associated with food waste have induced industries to reuse these materials in order to minimize food waste. In this sense, I have evaluated the bioactivity of two typologies of whey (freeze-dried cow's and liquid goat's whey) after subjecting these by-products to the fermentation process by lactic acid bacteria (*Lactobacillus plantarum* CECT 220, 221, 223, 748). Antifungal activity performed against 30 mycotoxigenic fungi belonging to the *Penicillium*, *Aspergillus* and *Fusarium* genera has shown a satisfying inhibitory effect. The value of MIC for *Aspergillus* and *Fusarium* genera was included in a range from 1.95 to 62.5 g/L whereas for *Penicillium* genera was included in a range from 3.9 to 125 g/L. The value of MFC was contained between 15.6 and 250 g/L for *Aspergillus*, 62.5 and 250 g/L for *Penicillium*, 3.9 and 250 g/L for *Fusarium*. The antifungal activity could be correlated to the presence of phenolic compounds identified in cell-free-supernatant. Based on previously performed experiments, mycotoxigenic strains of *P. expansum* and *P. brevicompactum* were employed for studying the shelf-life improvement of bread.

A valuable increase in the shelf-life was obtained for pita bread prepared with fermented whey. In particular, after the inoculation of bread with *Penicillium,* a rise of 1-2 and 7-8 days for the two typologies of assayed whey

was compared to a positive control. Through a natural contamination, an increase in the preservation period of bread (until the 20th day) was observed.

Regarding the antimicrobial activity, by adding the 1% of freeze-dried fermented whey in bread preparation, a percentage reduction ranged between 12-23% was obtained, whereas with the replacement of 100% of water used in bread preparation with whey fermented by LAB, the percentage of reduction has the highest peak of increase ranged from 42 to 92% for the different typologies of test performed.

In addition, concerning freeze-dried whey, I have evaluated the antioxidant, antihypertensive, and iron binding activity of the permeate, consisting of small bioactive compounds purified on Centricon Amicon with a cut-off of 3 kDa. The results highlighted a radical cation scavenging activity ranging from 1.415 to 2.083 mmol trolox equivalents TE/per kg of dry weight, a percentage of iron binding capacity ranging between 23–55% and a percentage of ACE inhibitory activity ranging between 67–85%. The optimal biological activity was obtained from whey fermented by *L. plantarum* 220 for all the assays performed, except for the iron chelating activity. These results confirm the interesting use of lactic acid bacteria as a source of new natural preservatives, which can contribute to the maintenance of food quality and safety. The obtained results support the hypothesis of using whey as a functional ingredient to improve food preservation and its potential use in nutraceutical formulation.

LIST OF PUBLICATIONS:

Paper I

Rodríguez-Carrasco, Y., Izzo, L., Gaspari, A., Graziani, G., Mañes, J., & Ritieni, A. (2018) **Simultaneous Determination of AFB1 and AFM1 in Milk Samples by Ultra High Performance Liquid Chromatography Coupled to Quadrupole Orbitrap Mass Spectrometry.** *Beverages,***4***, 43; doi:10.3390/beverages4020043*.

Paper II

Izzo, L., Rodríguez-Carrasco, Y., Gaspari, A., Graziani, G., Mañes, J., & Ritieni, A**. Development of a multi-residue method for the simultaneous determination of mycotoxins and veterinary drug residues in milk using UHPLC- HRMS analysis.** Manuscript draft ready for submission.

Paper III

Izzo, L.,* Luz, C., Graziani, G., Gaspari, A., Ritieni, A., Mañes, J., & Meca, G. (2018). **Evaluation of biological and antimicrobial properties of freeze-dried whey fermented by different strains of *Lactobacillus plantarum***. *Food & Function. DOI: 10.1039/c8fo00535d*

Paper IV

Izzo, L.,* Luz, C., Ritieni, A., Meca, G. **Short Communications: Antifungal activity of goat'ssweet whey subjected to the fermentation process by lactic acid bacteria.** *Journal of Diary Science*. Submitted.

Paper V

Luz, C., Izzo, L., Ritieni, A., Mañes, J., & Meca, G. **Whey hydrolysis through an enzymatic process, comparison with lactic acid bacteria in the shelf-life increase of bread.** Manuscript draft ready for submission.

Paper VI

Izzo, L.,* Luz, C., Ritieni, A., Meca, G. **Effect of sweet whey fermented by *Lactobacillus plantarum*: a promising approach to increase the shelf-life.** Manuscript draft ready for submission.

Other publications:

1. Tenore, G. C., Campiglia, P., Ciampaglia, R., Izzo, L., & Novellino, E. (2017). **Antioxidant and antimicrobial properties of traditional green and purple "Napoletano" basil cultivars (Ocimum basilicum L.) from Campania region (Italy).** *Natural product research, 31 (17), 2067-2071.*

2. Castaldo, L., Graziani, G., Gaspari, A., Izzo, L., Luz, C., Mañes, J., & Ritieni, A. (2018). **Study of the Chemical Components, Bioactivity and Antifungal Properties of the Coffee Husk.** *Journal of Food Research, 7 (4), 43*.

3. Rodríguez-Carrasco, Y., Izzo, L., Gaspari, A., Graziani, G., Mañes, J., & Ritieni, A. (2018). **Urinary levels of enniatin B and its phase I metabolites: First human pilot biomonitoring study.** Food and Chemical Toxicology, *118 (2018) 454–459.*

4. Castaldo L., Rodríguez-Carrasco, Y., Izzo, L., Gaspari, A., Graziani, G. & Ritieni, A. **Target analysis and retrospective screening of multiple mycotoxins in pet food using UHLPC-Q-Orbitrap HRMS**. Manuscript draft ready for submission.

LIST OF ABBREVIATIONS:

ADI Acceptable daily intake

AFB1 Aflatoxin B1

AFB2 Aflatoxin B2

AFG1 Aflatoxin G1

AFG2 Aflatoxin G2

AFM1 Aflatoxin M1

AFs Aflatoxins

BEA Beauvericin

CECT Spanish Type Culture Collection

CFS Cell-free supernatant

DMSO Dimethyl sulfoxide

DON Deoxynivalenol

DPPH Diphenyl-1-picrylhydrazyl

dSPE Dispersive Solid-Phase Extraction

EDI Estimated daily intake

EN A Enniatin A

EN A1 Enniatin A1

EN B Enniatin B

EN B1 Enniatin B1

ENs Enniatins

ESI Electrospray ionization

ESI-MS Electrospray ionization mass spectrometry

EU European Union

FB1 Fumonisin B1

FB2 Fumonisin B2

FBs Fumonisins

GRAS Generally recognized as safe

HCl Hydrochloric acid

HCOOH Formic acid

IARC International Agency for Research on Cancer

KCl Potassium chloride

LABs Lactic acid bacteria

LC Liquid-chromatography

LC-MS/MS Liquid chromatography tandem mass spectrometry

LOAEL Lowest observed adverse effect level

LOD Limit of Detection

LOQ Limit of Quantification

ME Matrix effects

MFC Minimum fungicidal concentration

$MgSO_4$ Magnesium sulfate

MIC Minimum inhibitory concentration

MRM Multiple reactions monitoring

MS Mass spectrometry

MS/MS Tandem mass spectrometry

MW Molecular weight

Na_2SO_4 Sodium sulfate

NaCl Sodium chloride

NaH_2PO_4 Monosodium phosphate

$NaHCO_3$ Sodium bicarbonate

NaOH Sodium hydroxide

ND No detected levels

NIV Nivalenol

OTA Ochratoxin A

PBS Phosphate buffer saline

PDA Potato dextrose agar

PDB Potato dextrose broth

PLA Phenyllactic acid

PSA Primary secondary amine

pTDI Provisional tolerable daily intake

PTFE PolytetrafluoroethyleneGRA

Qq Linear ion trap

QqQ Triple-quadrupole

QuEChERS quick, easy, cheap, effective, rugged and safe

R^2 Coefficient of determination

RSD Relative standard deviation

RSDr Repeatability relative standard deviation

RSDR Reproducibility relative standard deviation

RT Retention time

S/N Signal-to-noise ratio

TCs Thricothecenes

TDI Tolerable daily intake

TTA Total titratable acidity

ZEA Zearalenone

α-ZAL α-Zearalanol

α-ZOL α-Zearalenol

β-ZOL β-Zearalenol

LIST OF TABLES AND FIGURES

TABLE OF CONTENTS

INTRODUCTION

- **Objectives of the project**

The overall goals of this Ph.D. project were: (i) the development a multi-residue method for the simultaneous determination of milk contaminants including mycotoxins and veterinary drug residues, throughout a UHPLC Q-Orbitrap HRMS analysis, to investigate the occurrence in milk samples; (ii) a study regarding the potential bioactivity of dairy waste products, particularly the whey, in order to reuse this by-products in the bread preparation replacing it with the chemical preservatives.

- **Brief description of the outline of the thesis**

A brief description of my Ph.D. project and its main objectives was reported in this paragraph. The thesis is subdivided in two section; in the first part, the SECTION I, I have reported the experiments performed on milk to optimize a method for the simultaneous investigation of veterinary drug residues and mycotoxins which occurrence in milk. In this section, there are two chapters; *chapter 1,* in which there is an introduction about the production and the consumption of milk and the contaminants that could be found in it, such as mycotoxins and veterinary drug residues. Furthermore, I have reported the settlement in which was estabilished the maximum amounts of these contaminants in milk permitted in EU. Finally, I have described the selected extraction procedure and the technique that I have used to identify and quantify the contaminants in milk; in this case, I have employed a recent development of mass spectrometry: Q-Exactive Orbitrap. *Chapter 2* of this section contains the general and specific objectives that I have reached in this

first part. The latter chapter is subdivided in four subsections. The first specific objective includes the optimization of the chromatography and mass spectrometry conditions. The second specific objective considers the various test performed to obtain the optimization in the extraction process. The third specific objective describes the validation of method, and finally, the last specific objective includes the application of the validated method to 56 milk samples purchased in a supermarket in Naples, Campania. The last part of the first section contains the conclusions of the first section.

The second part, SECTION II of my project includes the study of whey in order to re-evaluate this by-product. I studied two types of whey: freeze-dried commercial cow's whey and liquid sweet goat's whey. I started my experiments evaluating the antifungal activity and the bioactivity of a commercial freeze-dried cow's whey and then, considering the promising results obtained, I decided to continue my experiments on whey coming from goat's milk. Also SECTION II is subdivided into two chapters. *Chapter 1* begins with a brief description of whey, its impact on the environment and the possibility to reuse this by-product after its fermentation by lactic acid bacteria. In this introductory part, I have described the potential biological activities awarded to the bioactive compounds derived from fermented whey (proteins, phenolic compounds, etc) and their possible application to increase the shelf-life of food. *Chapter 2* includes the general and specific objectives that I wanted to reach. *Chapter 2* is divided in turn in four subsections. The first specific objective of section II has been concerned the sample preparation for the experimental part, the evaluation of the macronutrients content of the two types of whey, the pasteurization of the liquid sweet whey adopted

to remove the microorganisms growth, and finally the further fermentation procedure was reported. In the specific objective 2, various assays of antifungal activity of fermented whey by four strains of *L. plantarum* (220, 221, 223 and 748) against thirty mycotoxigenic fungi were performed. In addition, the identification of phenolic compounds deriving from the fermentation process was carried out. The inhibition fungal growth assays on fermented whey gave us an initial overview on the antifungal activity of the different typologies of studied whey, confirming the interest in continuing the investigation on sweet whey fermented with *L. plantarum* 220, 221, and 223, given their effectiveness as antifungal agents. On the other hand, whey fermented by *L. plantarum* 748 was not taken into account, having shown a strongly inferior activity compared to the other ones. So, I decided to text the antifungal activity on a typical greek bread namely "pita bread". In specific objective 3, I have reported my experiments concerning the application of whey in bread preparation. I explained the contamination with two mycotoxigenic fungi (*P.expansum* and *P.brevicompactum*) given its recurrence in the contamination of bread, and I compared the results obtained by artificial contamination of bread with a natural contamination due to the normal environment. Finally, a microbiological assay on three typologies of bread was performed alongwith the evaluation of pH after the preservation period. In the last part of this section, the evaluation of the biological activity of freeze-dried whey including antioxidant, iron-binding and ACE inhibitory activity was presented. The conclusions were reported at the end of the section.

An increase of the shelf-life of pita bread was obtained replacing liquid whey in the fermentation process. Furthermore, whey could be a great candidate for the formulation of a potentially new nutraceutical.

❖ SECTION I

CHAPTER 1 - Milk

Milk is a beverage widely consumed in the world, not counting water, which provides high-quality nutrition to infants, children, and adults due to its high content of micro- and macronutrients. Generally, the intake of milk in most of the species concludes at the end of the weaning period, except in humans, in which milk consumption ongoing even during adulthood (Chon *et al.* 2016).

The Regulation (CE) No. 853/2004 of the European Parliament and of the Council of 29 April 2004, which establishes specific rules on hygiene for food of animal origin, defines:

"raw milk" as milk produced by the secretion of the mammary gland of farmed animals which has not been heated to more than 40°C and has not undergone any treatment having an equivalent effect;

"Dairy products" are defined as products resulting from the processing of raw milk or further processing of those products;

"Milk production holding" means an establishment where one or more farmed animals are kept to produce milk with a view to placing it on the market as food (EC No 853/2004).

According to the latest data reported by Food and Agriculture Organization of the United Nations (FAO), the European population has a significantly higher milk and dairy consumption than the global population, with an annual per capita data of 236.4 kg and 90 kg, respectively. Among the European countries, Italy is one of the highest milk and dairy consumers,

with an annual per capita consumption of 246.9 kg. In 2016 worldwide milk production was around 659 million tonnes, with an increase compared to 2006 of 14.7%. Italy milk production in 2016 was about 10 million tons, a constant value over the past 10 years (FAO, 2013). Cows' milk production and consumption are reported in table 1.

Table 1. Cows' milk production in the world and in Italy and milk consumption in Europe and Italy from 2006 to 2016 (FAOSTAT, 2018).

	Production (million tonnes)		**Consumption (kg/per capite)**	
Year	**World**	**Italy**	**UE**	**Italy**
2006	562.3	10.9	239.29	269.29
2007	574.7	10.6	240.51	258.15
2008	587.7	11.3	238.16	268.07
2009	591.7	10.6	238.34	258.28
2010	602.9	10.5	236.77	257.59
2011	617.3	10.5	238.2	260.78
2012	631.4	10.6	235.47	255.44
2013	636.6	10.4	236.41	246.88
2014	656.7	11.0	*n.d.*	*n.d.*
2015	666.7	11.1	*n.d.*	*n.d.*
2016	659.1	10.8	*n.d.*	*n.d.*
n.d. not reported				

The most commonly consumed types of milk is represented by bovine milk corresponding at 85% of the worldwide milk production, and the remaining part is divided between buffalo milk (11%), followed by goat (2.3%), sheep (1.4%) and camel milk (0.2%). The consumption of goat, sheep, buffalo, and camel milk are prevalent popular in other countries (Gerosa & Skoet, 2012). The nutritional composition of different types of milk is shown in table 2 and detailed composition of cow's milk is reported in table 3. Note that, several factors can influence the chemical composition of milk including

environmental conditions, animal nutritional status, lactation stage and animal species (Schwendel *et al.* 2015).

Table 2. Milk composition of selected animals' average % of whole milk.

Composition	Cattle	Buffalo	Sheep	Camel	Goat	Yat	Horse
Water	86.4	83	81.25	87	86.9	82	90
Fat	4.36	9.2	6.7	4.51	4.4	7.25	1.25
Proteins	3.37	4.86	5.17	3.64	3.4	6.2	2.15
Lactose	4.86	4.52	4.45	4.3	4.58	4.86	6.4
Minerals	2.15	0.8	0.9	0.77	0.8	0.8	0.4

Source: Compilated from multiple source

Table 3: Macro and micronutrient composition of cow's milk (CREA, 2018).

Composition	Values / 100 g
Energy (kcal)	63
Water (g)	87.6
Proteins (g)	3.3
of which:	
Casein (%)	80
Serum proteins (%)	20
Fat (g)	3.6
of which:	
Total saturated (g)	2.11
Total monounsaturated (g)	1.1
Total polyunsaturated (g)	0.12
Cholesterol (mg)	11
Carbohydrates (g)	4.7
Calcium (mg)	120
Potassium (mg)	150
Phosphorus (mg)	95
Iron (mg)	0.2
Magnesium (mg)	11
Zinc (mg)	0.3
Vitamin C (mg)	1
Vitamin B1 (mg)	0.04
Vitamin A (μg)	50
Vitamin E (mg)	0.07

Nowadays, the demand for milk protein and its products has risen substantially due to the impact on health and high biological effect. Milk protein may prevent bone breakdown, improve muscle movement, build-up muscles, raise satiety, control blood sugar, decrease the risk of some cancers and act on several biological disorder (Haug *et al.* 2007; Durazzo, 2017).

1.1 The main milk contaminants

Despite the benefits that milk provides from a nutritional point of view, it may also be a vehicle of food contaminants, such as mycotoxins and veterinary drug residues (VDs) due to nasty agricultures practices or improper usage of these drugs. In this sense, the first part of my project include the studies regarding the topics mentioned above.

1.1.1. Mycotoxigenic fungi

Toxigenic fungi are ubiquitous in nature and are considered to be among the most notable contaminants in foods concerning the impact on food safety, public health, and the national economy of several countries (Milićević *et al.* 2010, Adeyeye *et al.* 2016). The natural fungal flora related with foods is dominated by three genera of fungi: *Aspergillus*, *Fusarium*, and *Penicillium* which may be contaminated foods, feeds or the raw materials used to produce them, lead to the loss of food products. Foods contamination depends on different environmental factors such as moisture, temperature, and a matrix that favours the growth of a particular mould (Bhat *et al.* 2010). These three genera of filamentous fungi produce mycotoxins, secondary metabolites, capable of having acute toxic, carcinogenic, mutagenic, teratogenic, immunotoxic, and estrogenic effects in humans and animals (Sweeney *et al.* 1998; Pitt *et al.* 2000; Calvo *et al.* 2002; Richard, 2007).

Penicillium species

Penicillium represents a huge genus of fungi of which belonging to 150 different species, and about 50 or more common occurrence (Pitt *et al.* 1988). The variety of mycotoxins produced by *Penicillium* species is broader than other fungal genera. Several *Penicillium* species are among the most common agents of postharvest diseases affecting a wide range of fruits and vegetables. The major agent causing the blue mould rot of apples, pears and other fruits, is an example of the destructive postharvest pathogens of *Penicillium expansum*, that cause a large part of the economic losses that occur during storage and shipment. In addition to losses that are involved in postharvest, various *Penicillium* species may produce a variety of mycotoxins (Barkai-Golan & Paster, 2001). The *Penicillium* mycotoxins of importance in fruits and vegetables include patulin, ochratoxin A, citrinin, penicillic acid, cyclopiazonic acid, citreoviridin, penitrem; and other secondary metabolites, such as chaetoglobosins, communesins, roquefortine C, expansolides, janthitrems, paxillines, which may cause toxic responses in humans and animals. The most common mycotoxin of *P. expansum* is patulin, a relatively small molecule which may be produced by other *Penicillium* or by different *Aspergillus* species (Barkai-Golan, 2008).

Aspergillus species

Aspergillus is a group of moulds common in worldwide having a particular importance as organisms responsible for spoilage food and represent a large proportion of moulds found in industrial food (Yu *et al.* 2002, Taniwaki *et al.* 2018). *Aspergillus* species are more important in tropical and subtropical areas. Under favourable environmental conditions, *Aspergillus* species bring to changes in sensorial, nutritional and qualitative nature such as

pigmentation, discolouration, development of off odours and off flavours in foodstuffs and animal feeds (Perrone *et al.* 2007). As it has been reported by different recent papers, almost 50 species of *Aspergillus* genus showing the capacity to produce mycotoxins. Mycotoxins produced by *Aspergillus* species are able to grow up in whatsoever conditions, for example, *Aspergillus niger*, the most common species of *Aspergillus* responsible for post-harvest deterioration, occurring on a great variety of substrates. *Aspergillus flavus,* an opportunistic pathogen, is more frequently encountered in warm climates between 16 and 35 degree latitudes, and it is uncommon above 45 degree latitudes (Amaike and Keller, 2011; Ashiq, 2015; Pitt *et al.* 2000; William, 2018). As it is well-Known, the strains of *Aspergillus* are able to produce aflatoxins. The strains of *A. flavus*, *A. parasiticus* are producers of more than one aflatoxins like aflatoxins B1, B2, G1, and G2 and therefore these toxins can often be found in foods as mixtures, whereas closely related fungi such as *A. oryzae* and *A. sojae,* have never been shown to produce aflatoxins (Krishnan *et al* .2009). *Aspergillus* is responsible for a spectrum of disease, including allergic bronchopulmonary aspergillosis (ABPA), aspergilloma, chronic aspergillosis, and various forms of invasive aspergillosis, caused principally by *A. fumigatus* specifically for pulmonary, sinus, and central nervous system infection (Mwanda *et al.* 2005).

Fusarium species

Fusarium is regarded as one of the most important mycotoxigenic fungi genera. The *Fusarium* species are the most common fungi found on cereals grown in the temperate regions of America, Europe and Asia (EFSA, 2002a). *Fusarium* species can produce three of the most important classes of mycotoxins ones based on occurrence and toxicity: fumonisins (FBs), zearalenone (ZEA), and trichothecenes (deoxynivalenol (DON), nivalenol

(NIV), T-2 and HT-2 toxins), whereas *Fusarium* genera are also able to produce emerging mycotoxins, like fusaproliferin (FUS), beauvericin (BEA), enniatins (ENNs) and moniliformin (MON), which are more recently discovered and less studied (Summerell and Leslie, 2011; Escrivá *et al.* 2015). *Fusarium* mycotoxins are widely distributed in the food chain, showing a high occurrence especially in corn and wheat, the main sources of food intake of these toxins, but they can also found in other products including rice, barley, oats, potato and fodder (Cendoya *et al.* 2018). Generally, infect of *Fusarium* in cereals occurs in the field mainly before harvest. To this end, various actions must be carried out during the cultivation, harvesting and storage step and the application of agreeable agricultural practices, collection and storage (Nesic *et al.* 2014). As regarding the toxicity, DON, the most commonly detected *Fusarium* mycotoxin in cereal grains, was related to deleterious health effects such as malnutrition, endocrine dysfunction, anorexia, weight loss, and immune alterations (Pestka, 2010) but is non-classifiable as carcinogenic to humans (IARC, 1993).

1.1.2. Mycotoxins

Mycotoxins are secondary toxic low weight metabolites which are produced by several species of fungi, including *Aspergillus, Penicillium*, and *Fusarium* genera, but also *Trichoderma, Trichothecium* and *Alternaria* are important as food contaminants or pathogens for plants (Flores-Flores *et al.* 2015; Bhat *et al.* 2010). These fungi are able to contaminate agricultural products and to produce mycotoxins under favourable conditions, during pre- and post-harvest practices, processing and storage steps. The production of mycotoxins is stimulated by certain environmental factors: therefore the extent of contamination will differ with geographic location, agricultural methods and the susceptibility of commodities to the penetration of fungi

during storage and processing periods (Alshannaq *et al.* 2017). Contamination of food and feed with mycotoxins is a worldwide problem. In fact, the Food and Agriculture Organization (FAO) has estimated that a quarter of the world's crops are contaminated with mycotoxins, and it has a major economic impact (FAO, 2003). Moreover, mycotoxins are of significant public health concern, based on their high toxic profile. Mycotoxins may cause different toxic effects in animal and humans including carcinogenic, mutagenic, teratogenic, immunotoxic, estrogenic effects and mycotoxicoses (Schatzmayr*et al* 2013; Streit *et al.* 2013; Urusov *et al.* 2015). Some of the most important mycotoxins are aflatoxins, fumonisins, trichothecenes, ochratoxins, sterigmatocystins (STCs), and zearalenones (ZEAs) which will be further described in the next paragraph (Wu *et al.* 2014a).

Aflatoxins

Aflatoxins are a subgroup of toxins produced by different *Aspergillus* strains includes: *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus nomius*, *Aspergillus pseudotamarii*, and *Aspergillus bombycis* (Amaike and Keller, 2011; Becker-Algeri *et al.* 2016). Structurally, are a group of 20 related polycyclic structures belonging to the furanocoumarin class of compounds. Aflatoxins are classified in four major group: B1, B2, G1, and G2 with respect to the structural difference on their blue (B) or green (G) fluorescence under UV light and their relative chromatographic mobility (Yu, 2012). Apart from these, in mammals that consumed a diet contaminated with mycotoxins, the metabolism of aflatoxins B1 and B2 bring to the production of aflatoxins M1 and M2, which are metabolized and then transferred to animal-derived food. Aflatoxin B1 conversion to aflatoxin M1 involves the hydroxylation of the tertiary carbon of the difuranocoumarin ring, and the hydroxyl group increases water solubility, allowing a rapid excretion in urine, milk, and

feces. The rate of transformation of aflatoxin B1 in their metabolite M1 was up to 6 %, and this value is influenced by various factors including season, environmental conditions, geographic location, and diversity of the farming systems (Becker-Algeri *et al.* 2016). In fact, several studies reported that milk produced during the cold season was more contaminated than milk produced during the warm season, owing to the favourable conditions for fungal growth in cattle feed that had been stored for longer times during the cold season (Meat *et al.* 2008, Pleadin *et al.* 2015; Gnonlonfin *et. al* 2013). Generally, aflatoxin M1 can be detected within 12 h after animals are fed an aflatoxin B1-contaminated feed, whereas in humans, the exposure to aflatoxin M1 is correlated to the milk consumed in the diet (Giovati *et al.* 2015).

Toxicity

Aflatoxins are one of the most potent toxic substances that occur naturally. The adverse effects of aflatoxins can be classified into acute and chronic toxicity. Acute toxicity is caused when large doses of aflatoxins are ingested through the feed. Chronic toxicity occurs after a long-term exposure to low or moderate concentrations of aflatoxins. Adverse effects of this class of toxins include various forms of aflatoxicosis after aflatoxin exposure in humans. Acute aflatoxicosis which results from a few exposures may result in death, whereas chronic aflatoxicosis causes immune suppression, undersides growth, and hepatocellular carcinoma (Kamala *et al.* 2018, Guterres *et al.* 2017; Klich, 2007). Among the mycotoxins identified so far, aflatoxins (AFs), are the most extensively studied and one of most important classes from a public health perspective and are classified as carcinogenic to humans (Group 1) by the International Agency for Research on Cancer, the specialized cancer agency of the World Health Organization (IARC, 2012).

Aflatoxins cause immunogenic, highly teratogenic, and mutagenic effects in humans and animals, retard the growth in experimental animals and have been reported to provoke hepatic cancer in humans (Do *et al.* 2007; Kew, 2013; Yu, 2012, Luzardo *et al.* 2016). The toxicity of the aflatoxins increases in the order G2, B2, G1 and B1. Extensive research on various animal species has shown that aflatoxins, especially aflatoxin B1, are potential carcinogens (De Ruyck *et al.* 2015; Wu *et al.* 2009; Liu *et al.* 2012). In particular, the aflatoxin metabolite that exerts the carcinogenic effects is aflatoxin B1-8,9-epoxide (AFBO), which is able to form adducts with proteins and DNA, and produce mutations (Woo *et al.* 2011). One of the most studied mutational effects of aflatoxin B1 involves the human p53 gene. In fact, is reported mutations in this gene in 50% of the people with hepatocellular carcinoma (Aguilar *et al.* 1993; De Giorgi *et al.* 2015). Other studies reported the correlation between hepatocellular carcinoma and aflatoxin exposure up to about 25% (Magnussen *et al.* 2013; Liu and Wu, 2010).

Ochratoxins

Ochratoxins are secondary fungal metabolites produced by two genera of fungi, *Aspergillus* and *Penicillium*. Chemically, they consist of an isocoumarin and a phenylalanine moiety linked by an amide bond. Ochratoxin comes in three secondary metabolite forms, A, B, and C. Ochratoxin A (OTA) is a chlorinated toxin, first described in 1965 (van der Merwe *et al.* 1965). The Ochratoxin B (OTB) is a nonchlorinated form of Ochratoxin A (OTA) and Ochratoxin C (OTC) is an ethyl ester form Ochratoxin A (Bayman and Baker *et al.* 2006; Marin *et al.* 2013; Jeswal & Kumar, 2015). OTA is concentrated in the grain husks, in barley, wheat, rye, maize and rice, legumes, cacao, spices, wines, and raisins, and it has also been described in cows' milk and pork products (Duarte *et al.* 2010). Due to it stability, OTA is not destroyed by

common food preparation procedures. Based on its high affinity for proteins like albumin, OTA accumulates in animal organs and may cause carryover of the contamination (Bayman and Baker *et al.* 2006).

Toxicity

Ochratoxins have been labelled as a carcinogen, nephrotoxic, hepatotoxic, teratogenic, and immunotoxic compounds, and have been linked to cancer in the human urinary tract. In humans, renal cancer can occur when the diet contains more than 70 μg/kg OTA per day (Reddy and Bhoola *et al.* 2010). Teratogenic effects have been documented in several animal models, including mouse, rat, hamster, rabbit, and chick, and reduced birth weight and craniofacial abnormalities were the most common manifestations (Heussner and Bingle *et al* .2015; Malir *et al.* 2013). In this sense, IARC has classified OTA in 2B group (possibly carcinogenic agent).

Exposure

The Committee established a PTWI of 112 ng/kg bw/week, based on the deterioration of renal function in pigs, for which the LOEL was 8 μg/kg bw/day, and application of a safety factor of 500 (FAO, 2003).

Fumonisins

Fumonisins were discovered in 1988, through their isolation from cultures of *Fusarium verticillioides*, a frequent worldwide contaminant of maize particularly in warm and wet regions. In addition to corn and corn-based foods, the occurrance of FB1 was also described in beer, beans, soybeans, rice, sorghum, and asparagus (Scott, 2012). Structurally, they contain a long hydroxylated hydrocarbon chain and tricarballylic acid, methyl, and amino groups, of which the amino group is important for biological activity

(Stockmann-Juvala and Savolainen, 2008). Fumonisins have been grouped into four major categories, the A, B, C, and P. The predominant molecular form produced by *Fusarium moniliforme* is namely fumonisin B1, which is also able to produce FB2 (the 10-deoxy FB1) and FB3 (the 5-deoxy FB1) in significantly lower quantities (Soriano *et al.* 2005). Even though various studies did not find FB1 contamination in milk (Scott *et al.* 1994; Richard and others, 1996), the transfer of these contaminants to milk has been previously confirmed (Maragos and Richard 1994; Gazzotti *et al.* 2009; Gonzalez-Peñas *et al.* 2015).

Toxicity

Fumonisins exposure has been linked to carcinogenic effects in human and animals including liver, esophageal and kidney cancer. In addition, pulmonary artery hypertrophy and porcine pulmonary edema in swine, equine leukoencephalomalacia in horses, and atherosclerosis in monkeys were reported(Marasas *et al.* 2004; Domijan, 2012; Raiola *et al.* 2015, Ahangarkani *et al.* 2014; Escrivá *et al.* 2015). As regarding the toxicity, the most toxic compound FB1 is classified by the IARC as a Group 2B possible carcinogen to humans (IARC, 2012).

Exposure

The Joint FAO/WHO Expert Committee on Food Additives established the maximum tolerable daily intake of fumonisins as 2 μg/kg body weight for FB1, FB2, and FB3, alone or in combination (Marasas *et al.* 2004, WHO, 2002).

Trichothecenes

Trichothecenes (TCs) are a complex group of mycotoxins, structurally related to sesquiterpenoids. Based on their structural features, they are

divided into four groups (A, B, C and D); among these classification type A is more toxic than type B. Several genera of fungi belonging to the *Fusarium, Stachybotrys, Myrothecium, Trichothecium, Trichoderma, Cephalosporium, Cylindrocarpon, Verticimonosporium,* and *Phomopsis* genera can produce trichothecenes (Scott, 1989). Generally, TCs type A are producing by *F. sporotrichioidies*, *F. poae* and *F. equiseti* and TCs type B are produced by species of *F. graminearum* and *F. culmorum*. Structurally, trichothecenes can be macrocyclic and nonmacrocyclic due to the presence of macrocylic ester or of an ester–ether bridge between C-4 and C-15. The principal TCs type A includes: toxins T-2 and HT-2, diacetoxyscirpenol (DAS), neosolaniol (NEO) which do not possess a ketone group in position C-8. TCs type B are characterized by the presence of a ketone group in this position. In the latter group are included deoxynivalenol (DON), nivalenol (NIV), and their acetylated derivatives: 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON) and X-fusrenone (FUS-X) (Krska *et al.* 2007). Even though milk is not considered a significant source of trichothecene toxins, high levels of feed and silage contamination can also result in significant levels present in milk. Recent studies have reported that animals ingesting feed contaminated by type A or B trichothecenes (DON or T-2 toxin) showed mycotoxin biotransformation and excretion in fluids such as milk, thus classifying these as a risk to human health (Danicke and Brezina, 2013). However, there are few reports of the natural occurrence of these toxic compounds and their metabolites in milk samples intended for human consumption (Signorini *et al.* 2012).

Toxicity

The main adverse effects of TCs are classified in acute and chronic disorders. An acute toxicity can result in nausea, vomiting, loss of appetite,

growth retardation, and diarrhea. Systemic toxic effects include remarkable immunosuppressive activity and cytotoxicity attributed to the strong inhibition of protein, RNA and DNA synthesis (Cope, 2018; McLaughlin *et al.* 2017; Konigs *et al.* 2009; Wan *et al.* 2015; Sobrova *et al.* 2010). Within the TCs, the HT-2 and T-2 toxins are known to produce the Food Poisonous Aleukia (ATA), characterized by sepsis, haemorrhages and general pancytopenia. The TCs have been classified as non-carcinogenic for humans (Group 3) according to the IARC (1993).

Exposure

The Joint FAO/WHO Expert Committee on Food Additives established the maximum tolerable daily intake of DON (including its acetylated forms 3-DON and 15-DON) as 1 μg/kg per body weight and 60 ng/kg bw/day for T2 and HT-2 toxins alone or in combination (FAO, 2004).

Zearalenone

Zearalenone (ZEA) is a toxin produced from fungi of the genus *Fusarium* like *F. graminearum*, *F. culmorum* and *F. equiseti*. Structurally, is a lactone mycotoxin similar to 17β-estradiol, and its main metabolites are alpha and beta Zearalenol. ZEA is mainly present in corn but the toxin has also been detected in wheat, barley, oats, sorghum, rye, rice, and soybean products (Minervini *et al.* 2005, Zinedine *et al.* 2007; Summerell *et al.* 2012).

Toxicity

ZEA possesses pronounced estrogenic effects and various studies have hypothesized its ability to competitively bind estrogen receptors, explaining the alterations of reproductive tract observed and its ability to impair fertility in mice, rats, guinea pigs, hamsters and rabbits, and domestic animals (De

Ruyck *et al.* 2015; Escriva *et al.* 2015; Zinedine *et al.* 2007). The Agency International for Cancer Research has classified Zearalenone in Group 3 as "not classifiable as a carcinogenic agent for humans ".

Exposure

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has established a temporary tolerable maximum level of daily intake for Zearalenone and its metabolites (including alpha Zearalenol) of 0.25 µg/kg per body weight (EFSA, 2016).

Enniatins

ENs (A, A1, B and B1) and BEA are cyclohexadepsipeptides composed of alternating residues of three N-methyl amino acids, commonly leucine, isoleucine and valine, and three hydroxy acids, typically hydroxyisovaleric acid. ENs are produced by *F. trincinctum, F. poae, F. sporotrichioides, and F. langsethiae* species, whereas BEA producers include *F. poae, F. langsethiae, F. verticillioides, F. sporotrichioides, F. sambucinum, F. proliferatum,* and *F. subglutinans.* Finally, the strains of *F. avenaceum* can produce both ENs and small amounts of BEA (Logrieco *et al.* 1998; Thrane, 2001). ENNs possess antifungal, antimicrobial, anthelmintic, herbicide phytotoxic and cytotoxic potential activity. BEA has demonstrated insecticidal activity against a broad spectrum of microorganisms and an antibacterial activity against human, animal and plants pathogenic bacteria with no selectivity between Gram-positive and Gram-negative bacteria (Ruiz *et al.* 2011).

Toxicity

The mycotoxins mentioned above have demonstrated cytotoxic effects and are also able to change the intracellular ionic homeostasis. The principal toxic action of ENNs is related to their ionophoric properties, by which ENNs are

capable of promoting the formation of stable lipophilic complexes with essential cations mono and divalent (Ca^{2+}, Na^{+}, K^{+}) and their transport through biological membranes, disrupting normal physiological concentrations (Uhlig *et al.* 2006; Firakova *et al.* 2007, Wang & Xu, 2012).

1.1.3. Classification of veterinary drugs

1.1.3.1. Antibiotics

Antibiotics are drugs extensively used in the treatment and prevention of bacterial diseases, which can kill microorganisms or inhibit their growth. Generally, this class of drugs was used for therapeutic purposes to protect animal health but also to prevent economic loss, ensuring the safe food supply (Sarmah *et al.* 2006; Du and Liu, 2012). To treat or prevent infections of animals, they are applied as drugs or feed additives. The use of antibiotics in food producing animals is widely adopted worldwide, despite their use for growth promotion in livestock production was banned in EU (EU, 2003; Tang *et al.* 2018, Hao *et al.* 2014; Courtheyn *et al.* 2002; Serratosa *et al.* 2006).

The widespread use of antibiotics is often employed for no-therapeutic purposes. The main concern of extended antibiotics use is the promotion of bacterial resistance, which can enhance the formation of single, cross- and multiple resistance in pathogens, commensal and environmental bacteria (Laxminarayan *et al.* 2013). The most frequently used antibiotics are tetracyclines, sulphonamides, β-lactams and macrolides (Etebu *et al.* 2016; Sarmah *et al.* 2006). Among veterinary drugs can be found compounds with growth promoting activities which are substances improving the growth rate and feed efficiency of domestic animals when are added to animal feed in sub-therapeutic doses over an extended period of time (Kools *et al.* 2008; Bártíková *et al.* 2016; Lozano and Trujillo, 2012; Ronquillo *et al.* 2017).

Penicillins

Penicillin was one of the first widely available antibiotics, coming into use in the early 1940s. Despite its remarkably nontoxic effects to humans, it has shown immunogenicity reactions, in its early versions. Approximately 4-11% of the human population are believed to be allergic to penicillin and correlated drugs, thus exposure to this class of drug via food animal residues puts them at risk for developing allergic reactions which may range from minor reactions, like a skin rash, to severe anaphylaxis (Dayan, 1993). In particular, 6 ng/mL corresponding to 10 IU (0.6 mg), of drug in milk can cause anaphylaxis (EMA, 2008; Baynes *et al.* 2016). In this sense, the MRL and tolerance for this drug in milk is less than this concentration in many jurisdictions such EU and Codex, having an MRL <of 4 ng/mL (JECFA, 2015).

Tetracyclines

Tetracyclines, in addition to bacterial infections usage, have been also approved in various cases to treat Mycoplasma in poultry. Generally, are used for as few as 3-5 days or as long as several weeks in feed or water in order to control or treat bacterial enteritis or pneumonia. Anaphylaxis to tetracyclines is uncommon compared to the beta-lactam drugs. Human exposure to minocycline, a subgroup of tetracycline, has been correlated to approximately 13% adverse effects of which a small number results in anaphylactic reactions (Winckler & Grafe, 2001; Kemper, 2008; Jamal*et al.* 2017).

Aminoglycosides

As regard aminoglycosides, gentamicin and neomycin were usually used in livestock. Predominantly, they were used for treatment of infectious keratoconjunctivitis caused by *Moraxella bovis* in cattle, or to treat or control bacterial enteritis in piglets or young chickens and turkeys. To date, there is no reported evidence of human consumption of meat or milk products containing tetracyclines or aminoglycosides having resulted in adverse reactions associated with aminoglycoside or tetracycline toxicity (Krause *et al.* 2016).

Sulfonamides

One of the oldest groups of antimicrobials used in food animal production for over 60 years were represented by sulfonamides, the third most commonly antimicrobial used in food animals behind tetracyclines and penicillins. The main reasons for the occurrence of sulfonamide residues could be improper feed mixing, failure to observe the proper withdrawal time, and improper cleaning of feed mixing equipment. In addition, excretion of sulfamethazine in the feces and urine could also cause recontamination of the environment in swine and poultry houses and results in residues in the next group of animals when cleaning was not properly done (Barani *et al.* 2015). Oral sulfachlorpyridazine and sulfamethazine are commonly administered to calves to treat diarrhea, although there are restricted efficacy data that support this use (Riviere and Papich, 2009). Occasionally, sulfonamides are utilized in the poultry industry to control coccidiosis. The use of sulfonamide drugs can bring to the adverse drug reactions such as skin reactions which can range from a mild rash to a severe toxidermia or epidermal toxic necrolysis. Acute liver injuries associated with cotrimoxazole are also frequently reported in humans (Choquet-Kastylevskey *et al.* 2002). However, blood dyscrasias including neutropenia,

hemolytic anemia, thrombocytopenia and pancytopenia have been associated with sulfonamide use in humans. Another concern associated with the ingestion of sulfonamides is thyroid cancer, in fact, sulfamethazine produced a dose-dependent increase in follicular cell adenomas of the thyroid gland in both male and female mice (Tijani *et al.* 2016).

Chloramphenicol

Chloramphenicol is an older antibiotic widely used in pet animals. This antibiotic has a broad-spectrum antimicrobial activity against Gram-positive and negative microorganisms, anaerobes and rickettsiae. Several animal-derived food products including milk, honey, and meat have been contaminated with chloramphenicol after the use of this at therapeutic doses (Hanekamp *et al.* 2003). Adverse effects include bone marrow suppression resulting in aplastic anemia and genotoxic, and possible carcinogen effects due to exposure of the mentioned drug (Lai *et al.* 2009). The restricted studies available on the carcinogenicity do not allow a definitive classification of the drug. Since only a small exposure to chloramphenicol may lead to aplastic anemia, of particular importance in veterinary medicine, people handling and administering the drug may also be at risk (Hanekamp *et al.* 2015). In this sense, Codex indicated in its risk management the following recommendations "there is no safe level of residues of chloramphenicol or its metabolites in food that represents an acceptable risk to consumers, competent authorities should prevent residues of chloramphenicol in food by not using chloramphenicol in food producing animals" (CAC, 2014). For this reason, chloramphenicol is listed in Table 2 of EU document No. 37/2010 as a list of prohibited substances. Based on EFSA reported data, the exposure to animal-derived food products contaminated with chloramphenicol at or below 0.3 mg/kg are unlikely to be a health

concern for aplastic anemia or reproductive or hepatotoxic effects (EFSA, 2014).

1.1.3.2. Antiprotozoals and anticoccidial drugs

The antiprotozoals are drugs that kills or inhibits the growth of organisms known as protozoans. Protozoans cause a variety of diseases, including malaria and Chagas' disease (Zamboni *et al.* 2015). Members of this drug class include: metronidazole, ipronidazole, ronidazole, dimetridazole, and tinidazole. Metronidazole is the most commonly studied compound of this group which is used extensively in companion animal veterinary medicine for a variety of indications (Stock *et al.* 2018; Riviere and Papich, 2013). Antiprotozoals and anticoccidial drugs are often employed in protozoal infections of farm livestock such as babesiosis, cryptosporidiosis, giardiasis, toxoplasmosis, trypanosomosis, coccidiosis. They are often incorporated into feedstuff to prevent or treat various diseases (Boxall *et al.* 2002). Generally, were labelled for the treatment of swine dysentery, histomoniasis in turkeys and recommended as a treatment for trichomoniasis in bulls (Larina *et al.* 2009). As regards the toxicity, there is enough evidence to consider metronidazole as an animal carcinogen, but insufficient for assert the same to humans (Bendensky *et al.* 2002). Protozoan parasites are able to cause abortion and infertility in domestic ruminants (Kaltungo and Musa, 2013).

1.1.3.3. Antiparasitic drugs

Ectoparasiticides and endectocides have a huge range of application in livestock and companion animals. The first class of antiparasitic veterinary drugs was used to control external parasites. Animals are infected by several arachnids and insects, like various ticks, keds, flies, lice and mites. These latter can cause important irritation leading to inadequate conditions such as

weight decrease or reduced milk yield, resulting in major economic losses (Rajput *et al.* 2006). Based on the chemical structure, ectoparasiticides can be classified into various groups: amidines, carbamate, nitroguanidines, organochlorines, organophosphates, pyrethroids, macrocyclic lactones. The endectocide class was applied for treatment of diseases caused by both internal and external parasites (Taylor *et al.* 2005). Generally, various parasitic species are vectors of diseases to humans, due to the transmission of infections to animals themselves, increasing the necessity to an effective control (Patz *et al.* 2000).

1.1.3.4. Anthelmintics

Anthelmintic drugs are a class of drugs used for the treatment of a broad range of infections caused by parasitic worms include flatworms (flukes and tapeworms) and roundworms which result in a considerable animals morbidity and mortality. Based on similar chemical structure and mechanism of action, anthelmintics are classified in different classes: macrocyclic lactones, pyrazinoisoquinolines, benzimidazoles, imidazothiazoles, tetrahydropyrimidines, salicylanilides, nitrophenolic, organophospates (Coles *et al.* 2002; Kaczala *et al.* 2016). The most important drug is represented by praziquantel, belonging to the pyrazinoisoquinolines sub-group (Doenhoff *et al.* 2008). Niclosamide, classified as salicylanilides, is effective against tapeworms, whereas closantel associated with rafoxanide acting against flukes (Lateef*et al.* 2008). The use of anthelmintics frequently leads to drug resistance development, due to the amino-acetonitrile derivatives of which monepantel is a member (Kaminsky *et al.* 2008).

1.1.3.5. NSAIDs

NSAIDs are non-steroidal anti-inflammatory drugs. Generally, they are employed in practical therapy to treat inflammatory conditions, fever, osteoarthritis and rheumatoid arthritis in domestic animals (Crofford *et al.* 2013; Lamano-Carvalho, 2007). The NSAID drugs used in veterinary medicine include acetylsalicylic acid, paracetamol, phenylbutazone, flunixin meglumine, ketoprofen, meloxicam, tolfenamic acid, carprofen, etodolac, meloxicam, deracoxib and firocoxib (Bergh and Budsberg, 2005; Chen *et al.* 2008).

1.1.3.6. Benzodiazepnes

Anxiolytic and sedative drugs called benzodiazepines are used to treat insomnia, behaviour problems and associated conditions in companion animals (Licata *et al.* 2008). The most common drug employed in the veterinary field is diazepam, used as sedative and anxiolytic in animals transport to prevent stress. Other drugs, prescibed especially to dogs and cats, are represented by alprazolam, chlordiazepoxide, clonazepam, lorazepam, oxazepam, and triazolam (Mota-Rojas *et al.* 2011).

1.1.3.7. Hormones

Hormones represent a class of drugs extensively employed in animals diseases. Corticosteroids are divided in nature, such as cortisol and cortisone, and synthetic drug including dexamethasone, prednisolone, methylprednisone, betamethasone. The main applications of this class of drugs are: asthma, rheumatoid disease, gastrointestinal, renal, cardiac, reproductive disorders, the treatment of sick animals control and the improvement of the growth rate of animals, serving as growth promoters, although this application was banned (Courtheyn *et al.* 2002; Hughes & Heritage, 2004; Castanon *et al.* 2007; Casewell *et al.* 2003). Therapeutic use of

hormones also includes treatment of companion animals suffering from endocrine dysfunction including diabetes and hypothyroidism (Rees *et al.* 2005).

1.2. Legislation of mycotoxins and veterinary drug residues in milk

Hence, to protect consumers' health, EU has set both the maximum residue limits (MRLs) for veterinary drugs and the maximum limit (MLs) for mycotoxins in Commission Regulation (EC) No. 37/2010 and Commission Regulation (EC), No. 165/2010, which amending Regulation (EC), No. 1881/2006, respectively (EU 165/2010; EU 37/2010). MRLs of veterinary drug residues permitted in milk are in the range from 0.05 μg/kg to 1500 μg/kg. As regards mycotoxins, AFM1, the principal hydroxylated AFB1 metabolite, is the only mycotoxin regulated for animal-derived products. The maximum limit of AFM1 in raw milk, heat-treated milk and milk for the manufacture of milk-based products was set at 0.05 μg/kg and restricted of up to 0.025 μg/kg for infant formulae and follow-on formulae, including infant milk and follow-on milk.

1.3. Analysis of food contaminants

Nowadays, food security is a growing concern for consumers, governments and producers due to the globalization of markets in which food is produced and distributed worldwide. Food alarms in recent years have generated great interest and concern leading to provide suitable protective measures to ensure consumers safety. Nowadays, food contaminants analysis is still a challenge due to the heterogeneity of the different dietary matrices, the different chemical structures of the analysed compounds and the low detection required levels. The use of a single analysis technique which includes a huge group of contaminants is an objective to be achieved,

however, the simultaneous extraction of a wide range of compounds is a critical step. Advances in the analysis of contaminants are based on the development of faster methods, able to detect different analytes at once, respectful of the environment, economic and adequate to achieve the proposed objectives in food, feed, tissues and biological fluids (De Saeger *et al.* 2016). Most of the analytical methods reported in literature consist of sampling, homogenization, extraction steps followed by a purification of the sample to eliminate unwanted matrix components, and finally, the separation and detection steps, usually carried out by means of a chromatographic technique in combination with a variety of detectors (Pereira *et al.* 2014). In this sense, several analytical methodologies were applied and tested for the efficient determination of a simultaneous huge group of contaminants such as QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) or dispersive liquid-liquid microextraction (DLLME); however, more conventional techniques such as solid phase extraction (SPE) or solid-liquid extraction (SLE) are still widely used.

1.3.1. QuEChERS

Currently, one of the most used solid-liquid extraction methods is the QuEChERS procedure, which allows the extraction and purification in the presence of inorganic salts. This method was initially used for the determination of pesticides (Anastassiades, 2003), but in recent years it is also being used for the extraction and purification of other compounds (Sospedra *et al.* 2010; Rodríguez-Carrasco *et al.* 2012; Azaiez *et al.* 2014). The QuEChERS sample preparation method has a number of advantages over traditional techniques, such as a high yield, the use of small amounts of organic solvent (non-chlorinated), which makes it a fast and economical method for the extraction of food contaminants (Arroyo-Manzanares *et al*. 2014a). This

methodology consists of an extraction based on the distribution of the analytes between an aqueous phase and a solvent, followed by a purification step of the organic phase through a dispersion process in solid phase (d-SPE). In the extraction phase, an organic solvent, usually acetonitrile, is used, and subsequently, a series of salts, magnesium sulfate or sodium sulphate and sodium chloride or sodium acetate are added to remove moisture from the sample and promote the distribution of the samples analytes towards the organic phase. The purification step is carried out by the addition of PSA (Primary and Secondary Amine) to remove polar pigments, organic acids and other products present in the matrix that could interfere with the analytes. Generally, PSA is used in combination with C_{18} to remove lipids, sterols and pigments. The QuEChERS method has been suffering modifications from your first application regarding the reagents used and their proportions, depending on the analytes and the matrices to study. It is currently a preparation technique samples widely used for a wide variety of compounds as residues of veterinary drug (Stubbings and Bigwood, 2009; Aguilera-Luiz *et al.* 2008), pesticide residues (Wilkowska and Biziuk, 2011; Hou *et al.* 2013), or mycotoxins (Cunha and Fernandes, 2010, Ferreira *et al.* 2012; Rodríguez-Carrasco *et al.* 2013).

1.4. Identification and quantification of contaminants

For the identification and quantification of contaminants, the chromatographic separation of the different analytes coupled to different types of detectors are required, particularly mass spectrometers because of the analysis of food and feed requires the use of sensitive and selective methods. The different chemical structure of compounds, as well as the variety of matrices analyzed, hinders the development of multi-compounds analytical methods. Therefore, the development of analytical methods which

use a single sample preparation and a single final determination is, nowadays, the goal to achieve.

1.4.1. Chromatographic techniques

Today, it is well recognized that liquid chromatography (LC) coupled to mass spectrometry (MS) detectors are the most widely applied instrumental technique for contaminants residue analysis in food for their ability to separate mycotoxins from similar and different chemical structures (Hird *et al.* 2014). Generally, immunochemical methods are specific for a single compound or a small group of structurally related compounds (Emon, 2011). During the last decade several methods based on LC-MS/MS for the identification and quantification of mycotoxins and veterinary drug residues in different matrices have been published (Zachariasova *et al*. 2010, Rasmussen *et al*. 2010, Zhao *et al.* 2015; Stanciu*et al.* 2017b).

1.4.2. Mass spectrometry techniques

Mass spectrometry is a powerful analytical tool used in the identification of compounds, to elucidate the molecular weight, chemical structure of molecules and quantify contaminants in complex matrices. It allows us to obtain the distribution of the molecules in accordance with its mass/charge ratio (m/z). In recent years the traditional acquisition in HRMS has evolved, allowing a wide range of tools to identify or quantify compounds. This evolution has been made possible by improvements in HRMS instrumentation with the introduction of new instruments including Q-TOF, Q-IMS-TOF, IT-TOF, Q-Orbitrap.

Nowadays, different types of mass spectrometers are available: mass spectrometers with triple quadrupole and linear ion trap, which have a resolution power lower than 10,000 (mass accuracy> 5 ppm) while a high resolution (HRMS) it can be defined as a resolution power of 10,000-100,000 with a mass accuracy of <5 ppm (Holčapek *et al.* 2012). High-resolution mode is recommended for qualitative analysis, whereas low-resolution mode achieves the best results in terms of sensitivity and dynamic range. Thus, selection between both operation modes depends on the objective of the analysis.

The Orbitrap mass analyzer is the first high-performance mass analyzer which employs trapping of ions in electrostatic fields, together with a sophisticated ion injection process, which enables high resolution, mass accuracy, and excellent sensitivity for addressing numerous analytical applications in both research and routine analysis. Orbitrap mass analyzers allow selection of different resolution values, typically corresponding to 17,500; 35,000; 70,000; 100,000; or up to 140,000 FWHM in modern instruments. The principle of Orbitrap is based on the Kingdon ion trap described in 1923, and commercially at the first time in 2005 (Eliuk & Makarov, 2015). The development of screening methods that include the analysis of a large number of mycotoxins simultaneously are currently carried out by the use of LC-HRMS.

HRMS has been employed for a long time as a screening tool in food and feed for the analysis of veterinary drug residues, pesticide residues and natural toxins. HRMS instruments present several advantages with respect to conventional systems in residue analysis, providing an unequivocal identification mainly based on the accurate mass measurements and the capability to increase selectivity. This is one of the most important

characteristics when trace amounts of hundreds of analytes have to be determined in complex samples, as in my experiment.

Thus, HRMS provides a robust, sensitive and selective analysis by allowing the exploration of mass spectra in full scan mode, obtaining more complete, detailed and accurate information about the composition of a sample. Consequently, this characteristic allows the analysis of unknown compounds, as well as the possibility of retrospective data analysis without the need to re-inject the sample and the ability to carry out structural elucidations of unknown or suspicious compounds when a standard with which to compare is not available (Senyuva *et al.* 2015). Given this assumption, the latter described technique has been selected to achieve the simultaneous determination of mycotoxins and veterinary drug residues in milk.

References:

- Adeyeye, S. A. (2016). Fungal mycotoxins in foods: A review. *Cogent Food&Agriculture*, *2*(1), 1213127.
- Aguilar, F., Hussain, S. P., & Cerutti, P. (1993). Aflatoxin B1 induces the transversion of G T in codon 249 of the p53 tumor suppressor gene in human hepatocytes. *Proceedings of the National Academy of Sciences*, *90*(18), 8586-8590.
- Aguilera-Luiz, M. M., Vidal, J. L. M., Romero-González, R., & Frenich, A. G. (2008). Multi-residue determination of veterinary drugs in milk by ultra-high-pressure liquid chromatography–tandem mass spectrometry. *Journal of Chromatography A*, *1205*(1-2), 10-16.
- Ahangarkani, F., Rouhi, S., Azizi, I.G., (2014). A review on incidence and toxicity of fumonisins. *Toxin Rev*. 33 (3), 95–100.
- Alshannaq, A., & Yu, J. H. (2017). Occurrence, toxicity, and analysis of major mycotoxins in food. *International journal of environmental research and public health*, 14(6), 632.
- Amaike, S., & Keller, N. P. (2011). *Aspergillus* flavus. *Annual review of phytopathology*, *49*, 107-133.
- Anastassiades, M., Lehotay, S. J., & Schenck, F. J. (2003). QuEChERS procedure for multi-residue pesticide analysis. *Journal of AOAC International*, *86*, 1-8.
- Arroyo-Manzanares, N., Huertas-Pérez, J. F. & Gámiz-Gracia, L. (2014). Control de micotoxinas en alimentos. *Boletín Graseqa* 7, 1303-1307.
- Ashiq, S. (2015). Natural occurrence of mycotoxins in food and feed: Pakistan perspective. *Comprehensive Reviews in Food Science and FoodSafety*, 14(2), 159-175.
- Azaiez, I., Giusti, F., Sagratini, G., Mañes, J., & Fernández-Franzón, M. (2014). Multi-mycotoxins analysis in dried fruit by LC/MS/MS and a modified QuEChERS procedure. *Food analytical methods*, *7*(4), 935-945.
- Barani, A., & Fallah, A. A. (2015). Occurrence of tetracyclines, sulfonamides, fluoroquinolones and florfenicol in farmed rainbow trout in Iran. *Food and agricultural immunology*, *26*(3), 420-429.
- Barkai-Golan, R. (2008). Penicillium mycotoxins. *In Mycotoxins in fruits and vegetables* (pp. 153-183).
- Barkai-Golan, R., &Paster, N. (Eds.). (2011). Mycotoxins in fruits and vegetables. *Elsevier*.

- Bártíková, H., Podlipná, R., & Skálová, L. (2016). Veterinary drugs in the environment and their toxicity to plants. *Chemosphere*, *144*, 2290-2301.
- Bayman, P., & Baker, J. L. (2006). Ochratoxins: a global perspective. *Mycopathologia*, *162*(3), 215-223.
- Baynes, R. E., Dedonder, K., Kissell, L., Mzyk, D., Marmulak, T., Smith, G., & Riviere, J. E. (2016). Health concerns and management of select veterinary drug residues. *Food and Chemical Toxicology*, *88*, 112-122.
- Becker-Algeri, T. A., Castagnaro, D., de Bortoli, K., de Souza, C., Drunkler, D. A., & Badiale-Furlong, E. (2016). Mycotoxins in bovine milk and dairy products: a review. *Journal of food science*, *81*(3), R544-R552.
- Bendesky, A., Menéndez, D., & Ostrosky-Wegman, P. (2002). Is metronidazole carcinogenic?. *Mutation Research/Reviews in Mutation Research*, *511*(2), 133-144.
- Bergh, M. S., & Budsberg, S. C. (2005). The coxib NSAIDs: potential clinical and pharmacologic importance in veterinary medicine. *Journal of veterinary internal medicine*, *19*(5), 633-643.
- Bhat, R., Rai, R. V., & Karim, A. A. (2010). Mycotoxins in food and feed: present status and future concerns. *Comprehensive reviews in food science and foodsafety*, *9*(1), 57-81.
- Boxall, A. B. A., Fogg, L. A., Blackwell, P. A., Blackwell, P., Kay, P., Pemberton, E. J., & Croxford, A. (2004). Veterinary medicines in the environment. In *Reviews of environmental contamination and toxicology* (pp. 1-91). Springer, New York, NY.
- CAC., 2014. Joint FAO/WHO Food Standard Program Codex Alimentarius Commission Thirty-eighth Session. Maximum Residue Limits (MRLs) and Risk Management Recommendations (RMRs) for Residues of Veterinary Drugs in Foods. Geneva, Switzerland. http://www.codexalimentarius.org/standards/vetdrugs/veterinary-drug-detail/en/?d_id¼69
- Calvo, A. M., Wilson, R. A., Bok, J. W., & Keller, N. P. (2002). Relationship between secondary metabolism and fungal development. *Microbiology and molecular biology reviews*, *66*(3), 447-459.
- Casewell, M., Friis, C., Marco, E., McMullin, P., & Phillips, I. (2003). The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. *Journal of antimicrobial chemotherapy*, *52*(2), 159-161.
- Castanon, J. I. R. (2007). History of the use of antibiotic as growth promoters in European poultry feeds. *Poultry science*, *86*(11), 2466-2471.

- Cendoya, E., Chiotta, M. L., Zachetti, V., Chulze, S. N., & Ramirez, M. L. (2018). Fumonisins and fumonisin-producing *Fusarium* occurrence in wheat and wheat by products: A review. *Journal of Cereal Science.*
- Chen, Y. F., Jobanputra, P., Barton, P., Bryan, S., Fry-Smith, A., Harris, G., & Taylor, R. S. (2008). Cyclooxygenase-2 selective non-steroidal anti-inflammatory drugs (etodolac, meloxicam, celecoxib, rofecoxib, etoricoxib, valdecoxib and lumiracoxib) for osteoarthritis and rheumatoid arthritis: a systematic review and economic evaluation.
- Chon, J. W., Jang, H. S., Park, J. H., Jeong, D., Kang, I. B., Kim, Y. J. & Kim, D. H. (2016). Nutritional Functions of Milk and Dairy Products in Improving Human Health. *Journal of Milk Science and Biotechnology*, *34*(3), 145-155.
- Choquet-Kastylevsky, G., Vial, T., & Descotes, J. (2002). Allergic adverse reactions to sulfonamides. *Current allergy and asthma reports*, *2*(1), 16-25.
- Coles, G. C. (2002). Sustainable use of anthelmintics in grazing animals. *The Veterinary Record*, *151*(6), 165-169.
- Commission Regulation (EU) No 165/2010 of February 2010 Amending Regulation (EC) No1881/2006 Setting Maximum Levels for Certain Contaminants in Foodstuffs as Regards Aflatoxins. 2010. Available online: https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A32010R0165 (accessed on 4 October 2018).
- Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin (Text with EEA relevance) Available online: http://data.europa.eu/eli/reg/2010/37(1)/oj
- Cope, R. B. (2018). Trichothecenes. In *Veterinary Toxicology (Third Edition)* (pp. 1043-1053).
- Courtheyn, D., Le Bizec, B., Brambilla, G., De Brabander, H. F., Cobbaert, E., Van de Wiele, M., & De Wasch, K. (2002). Recent developments in the use and abuse of growth promoters. *Analytica Chimica Acta*, *473*(1-2), 71-82
- Crofford, L. J. (2013). Use of NSAIDs in treating patients with arthritis. *Arthritis research & therapy*, *15*(3), S2.
- Cunha, S. C., & Fernandes, J. O. (2010). Development and validation of a method based on a QuEChERS procedure and heart-cutting GC-MS for determination of five mycotoxins in cereal products. *Journal of separation science*, *33*(4-5), 600-609.

- Dänicke, S., & Brezina, U. (2013). Kinetics and metabolism of the *Fusarium* toxin deoxynivalenol in farm animals: Consequences for diagnosis of exposure and intoxication and carry over. *Food and chemical toxicology*, *60*, 58-75.
- Dayan, A. D. (1993). Allergy to antimicrobial residues in food: assessment of the risk to man. *Veterinary microbiology*, *35*(3-4), 213-226.
- De Giorgi, U., Yuan, J., Moroni, M., Veronese, S., Sartore-Bianchi, A., Broggini, M., & Ruffini, P. A. (2015). Germ cell tumors overexpress the candidate therapeutic target cyclin B1 independently of p53 function. *The International journal of biological markers*, *30*(3), 275-281.
- De Ruyck, K., De Boevre, M., Huybrechts, I., & De Saeger, S. (2015). Dietary mycotoxins, co-exposure, and carcinogenesis in humans: Short review. *Mutation Research/Reviews in Mutation Research*, *766*, 32-41.
- De Saeger, S., Audenaert, K., & Croubels, S. (2016). Report from the 5th International symposium on mycotoxins and toxigenic Moulds: challenges and perspectives (MYTOX) held in Ghent, Belgium, May 2016.
- Do, J. H., & Choi, D. K. (2007). Aflatoxins: detection, toxicity, and biosynthesis. *Biotechnology and Bioprocess Engineering*, *12*(6), 585-593.
- Doenhoff, M. J., Cioli, D., & Utzinger, J. (2008). Praziquantel: mechanisms of action, resistance and new derivatives for schistosomiasis. *Current opinion in infectious diseases*, *21*(6), 659-667.
- Domijan, A. M., Kovac, S., & Abramov, A. Y. (2012). Impact of fumonisin B1 on glutamate toxicity and low magnesium-induced seizure activity in neuronal primary culture. *Neuroscience*, *202*, 10-16.
- Du, L., & Liu, W. (2012). Occurrence, fate, and ecotoxicity of antibiotics in agro-ecosystems. A review. *Agronomy for Sustainable Development*, *32*(2), 309-327.
- Duarte, S. C., Pena, A., & Lino, C. M. (2010). A review on ochratoxin A occurrence and effects of processing of cereal and cereal derived food products. *Food microbiology*, *27*(2), 187-198. Jeswal, P., & Kumar, D. (2015). Mycobiota and natural incidence of aflatoxins, ochratoxin A, and citrinin in Indian spices confirmed by LC-MS/MS. *International journal of microbiology*, *2015*.
- Durazzo, A. (2017). Milk: Bioactive Components and Role in Human Nutrition.

- EFSA 2002a. Opinion of the Scientific Committee on Food on *Fusarium* toxins. Part 6: Group evaluation of T-2 toxin, HT-2 toxin, nivalenol and deoxynivalenol. *EFSA Journal* 2011, 9, 2197.
- EFSA., 2014. Scientific opinion on chloramphenicol in food and feed. EFSA panel on contaminants in the food chain (CONTAM). European food safety authority, Parma, Italy. *EFSA Journal*. 12 (11), 3907
- EFSA Panel on Contaminants in the Food Chain (CONTAM). (2016). Appropriateness to set a group health-based guidance value for zearalenone and its modified forms. *EFSA Journal*, 14(4), e04425.
- Eliuk, S., & Makarov, A. (2015). Evolution of orbitrap mass spectrometry instrumentation.
- EMA, 2008. European Medicines Agency. Committee for Veterinary Medicinal Products. Penicillins Summary Report. *Canary Wharf, London*, UK
- Emon, J. M. V. (2001). Immunochemical applications in environmental science. *Journal of AOAC International*, *84*(1), 125-133.
- Escrivá, L., Font, G., & Manyes, L. (2015). In vivo toxicity studies of *Fusarium* mycotoxins in the last decade: a review. *Food and Chemical Toxicology*, 78, 185-206.
- Etebu, E., & Arikekpar, I. (2016). Antibiotics: classification and mechanisms of action with emphasis on molecular perspectives. *Int J Appl Microbiol Biotechnol Res*, *4*, 90-101.
- Europea, C. (2004). Regolamento (CE) N. 853/2004 del Parlamento Europeo e del Consiglio del 29 aprile 2004 che stabilisce norme specifiche in materia di igiene per gli alimenti di origine animale. *Gazz Uff*, 55-205.
- European Commission (EC), 2003. Regulation (EC) No. 1831/2003 of the European Parliament and of the Council of 22 September 2003 on Additives for Use in Animal Nutrition.
- FAO. Worldwide regulations for mycotoxins in food and feed in 2003. 2004. http://www.fao.org/documents/show_cdr.asp?url_file=/docrep/007/y5499e/y5499e0f.htm.
- Ferreira, I., Fernandes, J. O., & Cunha, S. C. (2012). Optimization and validation of a method based in a QuEChERS procedure and gas chromatography–mass spectrometry for the determination of multi-mycotoxins in popcorn. *Food Control*, *27*(1), 188-193.

- Firakova, S., Proksa, B., & Šturdíková, M. (2007). Biosynthesis and biological activity of enniatins. *Die Pharmazie-An International Journal of Pharmaceutical Sciences*, *62*(8), 563-568.
- Flores-Flores, M. E., Lizarraga, E., de Cerain, A. L., & González-Peñas, E. (2015). Presence of mycotoxins in animal milk: A review. *Food Control*, *53*, 163-176.
- Food and Agriculture Organization Food Balance Sheet. 2013. Available online: http://www.fao.org/faostat/en/#data/FBS (accessed on 30 September 2018).
- Food and Agricolture Organization Wordwide Regulations for Mycotoxins in Food and Feed in 2003, 2004. Available online: www.fao.org/docrep/007/y5499e/y5499e00.htm (accessed on 17 October 2018).
- Gazzotti, T., Lugoboni, B., Zironi, E., Barbarossa, A., Serraino, A., & Pagliuca, G. (2009). Determination of fumonisin B1 in bovine milk by LC–MS/MS. *Food Control*, *20*(12), 1171-1174.
- Gerosa, S., & Skoet, J. (2012). Milk availability. *Trends in production and demand and medium-term outlook*.
- Giovati, L., Magliani, W., Ciociola, T., Santinoli, C., Conti, S., & Polonelli, L. (2015). AFM1 in milk: physical, biological, and prophylactic methods to mitigate contamination. *Toxins*, *7*(10), 4330-4349.
- Gnonlonfin, G. J. B., Hell, K., Adjovi, Y., Fandohan, P., Koudande, D. O., Mensah, G. A., & Brimer, L. (2013). A review on aflatoxin contamination and its implications in the developing world: A sub-Saharan African perspective. *Critical reviews in food science and nutrition*, *53*(4), 349-365.
- Gonzalez-Peñas, E., Lopez-de-Cerain, A., Lizarraga, E., & ME, F. F. (2015). Presence of mycotoxins in animal milk: a review.
- Guterres, K., Silva, C., Giordani, C., Matos, C., Athayde, C., Dilkin, P., & Cleff, M. (2017). Outbreak of acute aflatoxicosis in dogs in the municipality of Pelotas/RS, Brazil. *Pesquisa Veterinária Brasileira*, *37*(11), 1281-1286.
- Hanekamp, J. C., & Bast, A. (2015). Antibiotics exposure and health risks: Chloramphenicol. *Environmental toxicology and pharmacology*, *39*(1), 213-220.
- Hanekamp, J. C., Frapporti, G., & Olieman, K. (2003). Chloramphenicol, food safety and precautionary thinking in Europe. *Environmental Liability*, *11*(6), 209-219.

- Hao, H., Cheng, G., Iqbal, Z., Ai, X., Hussain, H. I., Huang, L., & Yuan, Z. (2014). Benefits and risks of antimicrobial use in food-producing animals. *Frontiers in microbiology*, *5*, 288.
- Haug, A., Høstmark, A. T., &Harstad, O. M. (2007). Bovine milk in human nutrition–a review. *Lipids in health and disease*, *6*(1), 25.
- Heussner, A. H., & Bingle, L. E. (2015). Comparative ochratoxin toxicity: A review of the available data. *Toxins*, *7*(10), 4253-4282.
- Hird, S. J., Lau, B. P. Y., Schuhmacher, R., & Krska, R. (2014). Liquid chromatography-mass spectrometry for the determination of chemical contaminants in food. *TrAC Trends in Analytical Chemistry*, *59*, 59-72.
- Holčapek, M., Jirásko, R. & Lísa, M. (2012). Recent developments in liquid chromatography–mass spectrometry and related techniques. *Journal of Chromatography A* 1259, 3-15.
- Hou, X., Han, M., Dai, X., Yang, X., & Yi, S. (2013). A multi-residue method for the determination of 124 pesticides in rice by modified QuEChERS extraction and gas chromatography–tandem mass spectrometry. *Food Chemistry*, *138*(2-3), 1198-1205.
- Hughes, P., & Heritage, J. (2004). Antibiotic growth-promoters in food animals. *FAO Animal Production and Health Paper*, 129-152.
- International Agency for Research on Cancer, IARC. Evaluation of carcinogenic risks of chemical to humans. In Some Naturally-Occurring Substances: Food Items and Constituents; Heterocyclic Aromatic Amines and Mycotoxins; IARC: Lyon, France, 1993; Volume 56, p. 245.
- International Agency for Research on Cancer. Agents Classified by the IARC Monographs; IARC: Lyon, France,2012; Volume 1, p. 103.
- Jamal, M. (2017). Lincomycin and tetracycline resistance in poultry. Review.
- JECFA., 2015. Joint FAO/WHO expert committee on food additives. Summary and conclusions. In: Eighty-first Meeting (Residues of Veterinary Drugs), Rome, 17e26 November 2015.
- Kaczala, F., & E Blum, S. (2016). The Occurrence of Veterinary Pharmaceuticals in the Environment: A Review. *Current analytical chemistry*, *12*(3), 169-182.
- Kaltungo, B. Y., & Musa, I. W. (2013). A review of some protozoan parasites causing infertility in farm animals. *ISRN Tropical Medicine*, *2013*.

- Kamala, A., Shirima, C., Jani, B., Bakari, M., Sillo, H., Rusibamayila, N., & investigation team. (2018). Outbreak of an acute aflatoxicosis in Tanzania during 2016. *World Mycotoxin Journal*, *11*(3), 311-320.
- Kaminsky, R., Ducray, P., Jung, M., Clover, R., Rufener, L., Bouvier, J., & Gauvry, N. (2008). A new class of anthelmintics effective against drug-resistant nematodes. *Nature*, *452*(7184), 176.
- Kemper, N. (2008). Veterinary antibiotics in the aquatic and terrestrial environment. *Ecological indicators*, *8*(1), 1-13.
- Kew, M. C. (2013). Aflatoxins as a cause of hepatocellular carcinoma. *Journal of Gastrointestinal & Liver Diseases*, *22*(3).
- Klich, M. A. (2007). *Aspergillus* flavus: the major producer of aflatoxin. *Molecular plant pathology*, *8*(6), 713-722.
- Königs, M., Mulac, D., Schwerdt, G., Gekle, M., & Humpf, H. U. (2009). Metabolism and cytotoxic effects of T-2 toxin and its metabolites on human cells in primary culture. *Toxicology*, *258*(2-3), 106-115.
- Kools, S. A., Moltmann, J. F., & Knacker, T. (2008). Estimating the use of veterinary medicines in the European union. *Regulatory Toxicology and Pharmacology*, *50*(1), 59-65.
- Krause, K. M., Serio, A. W., Kane, T. R., & Connolly, L. E. (2016). Aminoglycosides: an overview. *Cold Spring Harbor perspectives in medicine*, *6*(6), a027029.
- Krishnan, S., Manavathu, E. K., &Chandrasekar, P. H. (2009). *Aspergillus*flavus: an emerging non-fumigatus*Aspergillus* species of significance. Mycoses, 52(3), 206-222.
- Krska, R., Welzig, E., & Boudra, H. (2007). Analysis of *Fusarium* toxins in feed. *Animal Feed Science and Technology*, *137*(3-4), 241-264.
- Lai, H. T., Hou, J. H., Su, C. I., & Chen, C. L. (2009). Effects of chloramphenicol, florfenicol, and thiamphenicol on growth of algae Chlorella pyrenoidosa, Isochrysis galbana, and Tetraselmis chui. *Ecotoxicology and Environmental safety*, *72*(2), 329-334.
- Larina, L., & Lopyrev, V. (2009). Application of Nitroazoles. In *Nitroazoles: Synthesis, Structure and Applications* (pp. 407-432). Springer, New York, NY.
- Lateef, M., Zargar, S. A., Khan, A. R., Nazir, M., & Shoukat, A. (2008). Successful treatment of niclosamide-and praziquantel-resistant beef tapeworm infection with nitazoxanide. *International Journal of Infectious Diseases*, *12*(1), 80-82.

- Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A. K., Wertheim, H. F., Sumpradit, N., & Greko, C. (2013). Antibiotic resistance—the need for global solutions. *The Lancet infectious diseases*, *13*(12), 1057-1098.
- Licata, S. C., & Rowlett, J. K. (2008). Abuse and dependence liability of benzodiazepine-type drugs: GABAA receptor modulation and beyond. *Pharmacology Biochemistry and Behavior*, *90*(1), 74-89.
- Liu, Y., & Wu, F. (2010). Global burden of aflatoxin-induced hepatocellular carcinoma: a risk assessment. *Environmental health perspectives*, *118*(6), 818.
- Liu, Y., Chang, C. C. H., Marsh, G. M., & Wu, F. (2012). Population attributable risk of aflatoxin-related liver cancer: systematic review and meta-analysis. *European Journal of Cancer*, *48*(14), 2125-2136.
- Logrieco, A., Moretti, A., Castella, G., Kostecki, M., Golinski, P., Ritieni, A., & Chelkowski, J. (1998). Beauvericin Production by *Fusarium*Species. *Applied and Environmental Microbiology*, *64*(8), 3084-3088.
- Lozano, M. C., & Trujillo, M. (2012). Chemical residues in animal food products: An issue of public health. *In Public Health-Methodology, Environmental and Systems Issues*. InTech.
- Luzardo, O. P., del Mar Bernal-Suárez, M., Camacho, M., Henríquez-Hernández, L. A., Boada, L. D., Rial-Berriel, C., & Díaz-Díaz, R. (2016). Estimated exposure to EU regulated mycotoxins and risk characterization of aflatoxin-induced hepatic toxicity through the consumption of the toasted cereal flour called "gofio", a traditional food of the Canary Islands (Spain). *Food and Chemical Toxicology*, *93*, 73-81.
- Magnussen, A., & Parsi, M. A. (2013). Aflatoxins, hepatocellular carcinoma and public health. *World Journal of Gastroenterology: WJG*, *19*(10), 1508.
- Malir, F., Ostry, V., Pfohl-Leszkowicz, A., & Novotna, E. (2013). Ochratoxin A: Developmental and reproductive toxicity—An overview. *Birth Defects Research Part B: Developmental and Reproductive Toxicology*, *98*(6), 493-502.
- Maragos, C. M., & Richard, J. L. (1994). Quantitation and stability of fumonisins B1 and B2 in milk. *Journal of AOAC International*, *77*(5), 1162-1166.
- Marasas, W. F., Riley, R. T., Hendricks, K. A., Stevens, V. L., Sadler, T. W., Gelineau-van Waes, J., ... & Allegood, J. (2004). Fumonisins disrupt sphingolipid metabolism, folate transport, and neural tube development in embryo culture and in vivo: a

potential risk factor for human neural tube defects among populations consuming fumonisin-contaminated maize. *The Journal of nutrition*, *134*(4), 711-716.

- Marin, S., Ramos, A. J., Cano-Sancho, G., & Sanchis, V. (2013). Mycotoxins: Occurrence, toxicology, and exposure assessment. *Food and Chemical Toxicology*, *60*, 218-237.
- McLaughlin C. S., & Feinberg, B. (2017). Biochemical mechanism of action of trichothecene mycotoxins. In *Trichothecene Mycotoxicosis Pathophysiologic Effects (1989)*(pp. 27-36). CRC Press.
- Mead, M. N. (2008). Contaminants in human milk: weighing the risks against the benefits of breastfeeding. *Environmental Health Perspectives*, *116*(10), A426.
- Milićević, D. R., Škrinjar, M., &Baltić, T. (2010). Real and perceived risks for mycotoxin contamination in foods and feeds: challenges for food safety control. *Toxins*, *2*(4), 572-592.
- Minervini, F., Giannoccaro, A., Cavallini, A., & Visconti, A. (2005). Investigations on cellular proliferation induced by zearalenone and its derivatives in relation to the estrogenic parameters. *Toxicology Letters*, *159*(3), 272-283.
- Mota-Rojas, D., Orozco-Gregorio, H., Gonzalez-Lozano, M., Roldan-Santiago, P., Martinez-Rodriguez, R., Sanchez-Hernandez, M., & Trujillo-Ortega, M. E. (2011). Therapeutic approaches in animals to reduce the impact of stress during transport to the slaughterhouse: A review. *International Journal of Pharmacology*, *7*(5), 568-78.
- Mwanda, O.W.; Otieno, C.F.; Omonge, E. Acute aflatoxicosis: Case report. E. Afr. Med. J. 2005, 82, 320–324.
- Nesic, K., Ivanovic, S., & Nesic, V. (2014). Fusarial toxins: secondary metabolites of *Fusarium* fungi. In Reviews of Environmental Contamination and Toxicology Volume 228 (pp. 101-120). *Springer, Cham.*
- Patz, J. A., Graczyk, T. K., Geller, N., & Vittor, A. Y. (2000). Effects of environmental change on emerging parasitic diseases. *International journal for parasitology*, *30*(12-13), 1395-1405.
- Pereira, V. L., Fernandes, J. O., & Cunha, S. C. (2014). Mycotoxins in cereals and related foodstuffs: A review on occurrence and recent methods of analysis. *Trends in Food Science & Technology*, *36*(2), 96-136.

- Perrone, G., Susca, A., Cozzi, G., Ehrlich, K., Varga, J., Frisvad, J. C., & Samson, R. A. (2007). Biodiversity of *Aspergillus* species in some important agricultural products. *Studies in mycology*, 59, 53-66.
- Pestka, J. J. (2010). Deoxynivalenol: mechanisms of action, human exposure, and toxicological relevance. *Archives of toxicology*, *84*(9), 663-679.
- Pitt, J. I. (2000). Toxigenic fungi and mycotoxins. *Britishmedicalbulletin*, *56*(1), 184-192.
- Pitt, J. I., & Hocking, A. D. (1997). *Penicillium*and related genera. In *Fungi and food spoilage* (pp. 203-338). *Springer, Boston, MA.*
- Pitt, J. I., Basilico, J. C., Abarca, M. L., & Lopez, C. (2000). Mycotoxins and toxigenic fungi. *Medical mycology*, 38(sup1), 41-46.
- Pleadin, J., Vulić, A., Perši, N., Škrivanko, M., Capek, B., & Cvetnić, Ž. (2015). Annual and regional variations of aflatoxin B1 levels seen in grains and feed coming from Croatian dairy farms over a 5-year period. *Food control*, *47*, 221-225.
- Raiola, A., Tenore, G. C., Manyes, L., Meca, G., & Ritieni, A. (2015). Risk analysis of main mycotoxins occurring in food for children: An overview. *Food and Chemical Toxicology*, *84*, 169-180.
- Rajput, Z. I., Hu, S. H., Chen, W. J., Arijo, A. G., & Xiao, C. W. (2006). Importance of ticks and their chemical and immunological control in livestock. *Journal of Zhejiang University Science B*, *7*(11), 912-921.
- Rasmussen, R. R., Storm, I. M. L. D., Rasmussen, P. H., Smedsgaard, J., & Nielsen, K. F. (2010). Multi-mycotoxin analysis of maize silage by LC-MS/MS. *Analytical and Bioanalytical Chemistry*, *397*(2), 765-776.
- Reddy, L., & Bhoola, K. (2010). Ochratoxins—Food contaminants: Impact on human health. *Toxins*, *2*(4), 771-779.
- Rees, D. A., & Alcolado, J. C. (2005). Animal models of diabetes mellitus. *Diabetic medicine*, *22*(4), 359-370.
- Richard, J. L., Meerdink, G., Maragos, C. M., Tumbleson, M., Bordson, G., Rice, L. G., & Ross, P. F. (1996). Absence of detectable fumonisins in the milk of cows fed *Fusarium* proliferatun (Matsushima) Nirenberg culture material. *Mycopathologia*, *133*(2), 123-126.
- Riviere, J. E., & Papich, M. G. (Eds.). (2013). Veterinary pharmacology and therapeutics. *John Wiley & Sons.*

- Rodríguez-Carrasco, Y., Berrada, H., Font, G., & Mañes, J. (2012). Multi-mycotoxin analysis in wheat semolina using an acetonitrile-based extraction procedure and gas chromatography–tandem mass spectrometry. *Journal of Chromatography A*, *1270*, 28-40.
- Rodríguez-Carrasco, Y., Ruiz, M. J., Font, G., & Berrada, H. (2013). Exposure estimates to *Fusarium* mycotoxins through cereals intake. *Chemosphere*, *93*(10), 2297-2303.
- Ronquillo, M. G., & Hernandez, J. C. A. (2017). Antibiotic and synthetic growth promoters in animal diets: review of impact and analytical methods. *Food Control*, *72*, 255-267.
- Ruiz, M. J., Franzova, P., Juan-García, A., & Font, G. (2011). Toxicological interactions between the mycotoxins beauvericin, deoxynivalenol and T-2 toxin in CHO-K1 cells in vitro. *Toxicon*, *58*(4), 315-326.
- Sarmah, A. K., Meyer, M. T., & Boxall, A. B. (2006). A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. *Chemosphere*, *65*(5), 725-759.
- Schatzmayr, G., & Streit, E. (2013). Global occurrence of mycotoxins in the food and feed chain: facts and figures. World Mycotoxin Journal, 6(3), 213-222.
- Schwendel, B. H., Wester, T. J., Morel, P. C. H., Tavendale, M. H., Deadman, C., Shadbolt, N. M., & Otter, D. E. (2015). Invited review: Organic and conventionally produced milk—An evaluation of factors influencing milk composition. *Journal of dairy science*, *98*(2), 721-746.
- Scott, P. M. (2012). Recent research on fumonisins: a review. *Food Additives & Contaminants: Part A*, *29*(2), 242-248.
- Scott, P. M., Delgado, T., Prelusky, D. B., Trenholm, H. L., & Miller, J. D. (1994). Determination of fumonisins in milk. *Journal of Environmental Science & Health Part B*, *29*(5), 989-998.
- Scott, P. M., Lombaert, G. A., Pellaers, P., Bacler, S., Kanhere, S. R., Sun, W. F., & Weber, D. (1989). Application of capillary gas chromatography to a survey of wheat for five trichothecenes. *Food Additives & Contaminants*, *6*(4), 489-500.
- Senyuva, H. Z., Gökmen, V. & Sarikaya, E. A. (2015). Future perspectives in Orbitrap™-high-resolution mass spectrometry in food analysis: a review. *Food Additives and Contaminants: Part A* 32, 1568-1606.
- Serratosa, J., Blass, A., Rigau, B., Mongrell, B., Rigau, T., Tortades, M., & Balagué, J. (2006). Residues from veterinary medicinal products, growth promoters and

performance enhancers in food-producing animals: a European Union perspective. *Rev Sci Tech*, *25*(2), 637-53.

- Signorini, M. L., Gaggiotti, M., Molineri, A., Chiericatti, C. A., De Basílico, M. Z., Basílico, J. C., & Pisani, M. (2012). Exposure assessment of mycotoxins in cow's milk in Argentina. *Food and chemical toxicology*, *50*(2), 250-257.
- Sobrova, P., Adam, V., Vasatkova, A., Beklova, M., Zeman, L., & Kizek, R. (2010). Deoxynivalenol and its toxicity. *Interdisciplinary toxicology*, *3*(3), 94-99.
- Soriano, J. M., Gonzalez, L., & Catala, A. I. (2005). Mechanism of action of sphingolipids and their metabolites in the toxicity of fumonisin B1. *Progress in lipid research*, 44(6), 345-356.
- Sospedra, I., Blesa, J., Soriano, J. M., & Mañes, J. (2010). Use of the modified quick easy cheap effective rugged and safe sample preparation approach for the simultaneous analysis of type A-and B-trichothecenes in wheat flour. *Journal of Chromatography A*, *1217*(9), 1437-1440.
- Stanciu, O., Juan, C., Miere, D., Loghin, F. & Mañes, J. (2017b). Analysis of enniatins and beauvericin by LC-MS/MS in wheat-based products. *CyTAJournal of Food*. doi.org/10.1080/19476337.2017.1288661
- Stock, M. L., Elazab, S. T., & Hsu, W. H. (2018). Review of triazine antiprotozoal drugs used in veterinary medicine. *Journal of veterinary pharmacology and therapeutics*, *41*(2), 184-194.
- Stockmann-Juvala, H., & Savolainen, K. (2008). A review of the toxic effects and mechanisms of action of fumonisin B1. *Human &experimental toxicology*, 27(11), 799-809.
- Streit, E., Schwab, C., Sulyok, M., Naehrer, K., Krska, R., & Schatzmayr, G. (2013). Multi-mycotoxin screening reveals the occurrence of 139 different secondary metabolites in feed and feed ingredients. *Toxins*, 5(3), 504-523.
- Stubbings, G., & Bigwood, T. (2009). The development and validation of a multiclass liquid chromatography tandem mass spectrometry (LC–MS/MS) procedure for the determination of veterinary drug residues in animal tissue using a QuEChERS (QUick, Easy, CHeap, Effective, Rugged and Safe) approach. *Analytica Chimica Acta*, *637*(1-2), 68-78.
- Summerell, B. A., & Leslie, J. F. (2011). Fifty years of *Fusarium*: how could nine species have ever been enough?. *Fungal Diversity*, *50*(1), 135.

- Sweeney, M. J., & Dobson, A. D. (1998). Mycotoxin production by *Aspergillus*, *Fusarium* and *Penicillium*species. *International journal of foodmicrobiology*, *43*(3), 141-158.
- Tang, K. L., Caffrey, N. P., Nóbrega, D. B., Cork, S. C., Ronksley, P. E., Barkema, H. W., & Ghali, W. A. (2017). Restricting the use of antibiotics in food-producing animals and its associations with antibiotic resistance in food-producing animals and human beings: a systematic review and meta-analysis. *The Lancet Planetary Health*, *1*(8), e316-e327.
- Taniwaki, M. H., Pitt, J. I., &Magan, N. (2018). *Aspergillus* species and mycotoxins: occurrence and importance in major food commodities. *Current Opinion in Food Science.*
- Taylor, M. A. (2005). Ectoparasiticides used in large animals. *The Merck Veterinary Manual., 9th edition. Merck and Co., Inc., Whitehouse Station, USA*, 2158-2165.
- Thrane, U., Adler, A., Clasen, P. E., Galvano, F., Langseth, W., Lew, H., & Ritieni, A. (2004). Diversity in metabolite production by *Fusarium* langsethiae, *Fusarium* poae, and *Fusarium* sporotrichioides. *International Journal of Food Microbiology*, *95*(3), 257-266.
- Tijani, J. O., Fatoba, O. O., Babajide, O. O., & Petrik, L. F. (2016). Pharmaceuticals, endocrine disruptors, personal care products, nanomaterials and perfluorinated pollutants: a review. *Environmental chemistry letters*, *14*(1), 27-49.
- Uhlig, S., Torp, M., & Heier, B. T. (2006). Beauvericin and enniatins A, A1, B and B1 in Norwegian grain: a survey. *Food Chemistry*, *94*(2), 193-201.
- Van der Merwe, K. J., Steyn, P. S., Fourie, L., Scott, D. B., & Theron, J. J. (1965). Ochratoxin A, a toxic metabolite produced by *Aspergillus* ochraceus Wilh. *Nature*, *205*(4976), 1112.
- Wan, D., Wang, X., Wu, Q., Lin, P., Pan, Y., Sattar, A., & Yuan, Z. (2015). Integrated transcriptional and proteomic analysis of growth hormone suppression mediated by trichothecene T-2 toxin in rat GH3 cells. *Toxicological Sciences*, *147*(2), 326-338.
- Wang, Q., & Xu, L. (2012). Beauvericin, a bioactive compound produced by fungi: a short review. *Molecules*, *17*(3), 2367-2377.
- Wilkowska, A., & Biziuk, M. (2011). Determination of pesticide residues in food matrices using the QuEChERS methodology. *Food Chemistry*, *125*(3), 803-812.
- William J. Steinbach 244: *Aspergillus* SpeciesPrinciples and Practice of Pediatric Infectious Diseases (Fifth Edition), 2018, Pages 1238-1244.e2

- Winckler, C., & Grafe, A. (2001). Use of veterinary drugs in intensive animal production. *Journal of soils and sediments*, *1*(2), 66.
- Woo, L. L., Egner, P. A., Belanger, C. L., Wattanawaraporn, R., Trudel, L. J., Croy, R. G., ... & Wogan, G. N. (2011). Aflatoxin B1-DNA adduct formation and mutagenicity in livers of neonatal male and female B6C3F1 mice. *Toxicological Sciences*, *122*(1), 38-44.
- Wu, F., Groopman, J. D., & Pestka, J. J. (2014). Public health impacts of foodborne mycotoxins. *Annual review of food science and technology*, 5, 351-372.
- Wu, H. C., Wang, Q., Yang, H. I., Ahsan, H., Tsai, W. Y., Wang, L. Y., & Santella, R. M. (2009). Aflatoxin B1 exposure, hepatitis B virus infection, and hepatocellular carcinoma in Taiwan. *Cancer Epidemiology and Prevention Biomarkers*, *18*(3), 846-853.
- Yu, J. (2012). Current understanding on aflatoxin biosynthesis and future perspective in reducing aflatoxin contamination. *Toxins*, *4*(11), 1024-1057.
- Yu, J.; Bhatnagar, D. & Ehrlich, K.C. (2002). Aflatoxinbiosynthesis. *Revista Iberoamericana de Micología*, Vol. 19, pp. 191-200
- Zachariasova, M., Lacina, O., Malachova, A., Kostelanska, M., Poustka, J., Godula, M., & Hajslova, J. (2010). Novel approaches in analysis of *Fusarium* mycotoxins in cereals employing ultra performance liquid chromatography coupled with high resolution mass spectrometry. *Analytica Chimica Acta*, *662*(1), 51-61.
- Zamboni, D. S., & Lima-Junior, D. S. (2015). Inflammasomes in host response to protozoan parasites. *Immunological reviews*, *265*(1), 156-171.
- Zhao, Z., Liu, N., Yang, L., Deng, Y., Wang, J., Song, S., & Hou, J. (2015). Multi-mycotoxin analysis of animal feed and animal-derived food using LC–MS/MS system with timed and highly selective reaction monitoring. *Analytical and bioanalytical chemistry*, *407*(24), 7359-7368.
- Zinedine, A., Soriano, J. M., Molto, J. C., & Manes, J. (2007). Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: an oestrogenic mycotoxin. *Food and chemical toxicology*, *45*(1), 1-18.

CHAPTER 2 - Objectives section I

2.1.General Objective

The aim of this part of my thesis work was to develop a multi-residue method for the simultaneous determination of mycotoxins (*n=46*) and veterinary drug residues (*n=61*) in milk to investigate the occurrence of the before mentioned compounds in 56 milk samples in order to monitor the exposure in the global population, as well as in susceptible groups such as children.

2.2. Specific Objectives:

1. Optimization of spectrometric (Q-Exactive Orbitrap HRMS) and chromatographic (UHPLC) conditions for the simultaneous determination of mycotoxins and veterinary drug residues in milk;
2. Development of a multi-residue method based on QuEChERS procedure for the extraction and purification of veterinary drugs and mycotoxins in milk;
3. Validation of the analytical method in accordance with the regulation 2002/657/CE on the performance of analytical methods;
4. Application of validated method to investigate the occurrence of the before mentioned compounds in 56 milk samples purchased in Campania, Italy.

SPECIFIC OBJECTIVES 1

Optimization of spectrometric (Q-Exactive Orbitrap HRMS) and chromatographic (UHPLC) conditions for the simultaneous determination of mycotoxins and veterinary drug residues in milk.

Materials and Methods

Chemicals and materials

Methanol (MeOH), acetonitrile (AcN) and (LC-MS grade) were acquired from Merk (Darmstadt, Germany). Formic water acid (mass spectrometry grade) and ammonium formate (analytical grade) were purchased from Fluka (Milan, Italy). Syringe filters with polytetrafluoroethylene membrane (PTFE, 15 mm, diameter 0.22 μm) were supplied by Phenomenex (Castel Maggiore, Italy). Glass amber vials with septum screw caps were supplied by Agilent Technologies (Milan, Italy).

The standards of veterinary drugs and mycotoxins (purity >98%) were purchased from Sigma Aldrich (Milan, Italy). Veterinary drugs were selected from those with lower MRLs or prohibited in milk in accordance with EC Regulation No. 37/2010. The standard of veterinary drugs included: metronidazole, clenbuterol hydrochloride, meloxicam, dexamethasone, amoxicillin, ampicillin, benzylpenicillin procaine, ceftiofur, danofloxacin, monensin sodium, sulfadimidine, dapsone, chloramphenicol, abamectin, amitraz, cyhalothrin, cipermethrin, trichlorfon, colchicine, imidocarb, doramectin, ivermectin, eprinomectin and deltamethrin. The standard of mycotoxins included: aflatoxins (AFM1, AFB1, AFB2, AFG1 and AFG2), ochratoxin A (OTA), fumonisins (FB1 and FB2), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-AcDON), 15-acetyldeoxynivalenol (15-AcDON),

nivalenol (NIV), HT-2 toxin, T2 toxin, neosolaniol (NEO), diacetoxyscirpenol (DAS), fusarenon-X (FUS-X), zearalenone (ZON), α-zearalenol (α-ZOL), ß-zearalenol (ß-ZOL), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL), zearalanone (ZAN), beauvericin (BEA), enniatins (ENNA, ENNA1, ENNB and ENNB1), alternariol (AOH) and alternariolmonomethyl ether (AME).

To lack of analytical standards, the identification of veterinary drug (*n=37*) including: albendazole, betamethasone, carprofen, cefalexin, cefazolin, chlorpromazine, chlortetracycline, cloxacillin, cypermethrin, decoquinate, diazinon, dicloxacillin, difloxacin, enrofloxacin, febantel, fenbendazole, florfenicol, flumequine, levamisole, lyncomycin, marbofloxacin, mebendazole, methylprednisolone, oxacillin, oxfendazole, oxolinic acid, oxytetracycline, prednisolone, rifaximin, ronidazole, spiramycin, tetracycline, thiamphenicol, tilmicosinand mycotoxins (*n=16*) including: chaetoglobosin a, cyclopiazonic acid, deoxynivalenol-3-glucoside, gliotoxin, hydrolyzed fumonisin B1, hydrolyzed fumonisin B2, penicillic acid, tentoxin, AFM2, OTAb, α-OTA, sterigmatocystin, FB3, deepoxy-deoxynivalenol (DOM), t2-trialwere carried out by a post-target screening.

Individual standard solutions were prepared by diluting 1 mg of each analyte in 1 mL of solvent (MeOH, EtOH, ACN or DMSO) based on the specifications of the supplier. Three stock solutions were prepared:stock solution 1 (including all the mycotoxins, except aflatoxins), stock solution 2 (including aflatoxins, clenbuterol hydrochloride and prohibited substances in milk) and stock solution 3 (including the rest of the studied veterinary drugs), were prepared by taking a defined volume from each one of the individual standard solutions and then performing adequate dilution to reach appropriate working standard solutions for spiking experiments. The working standard solutions were prepared at 25, 250, 1250 ng/L (from stock

1), at 2.5, 5, 10 ng/L (from stock 2), and at 0.2, 2, 10 mg/L (from stock 3). All solutions were stored at −20°C in screw-capped glass vials.

UHPLC-Q-Orbitrap HRMS analysis

Analysis were performed by using an UHPLC instrument (Dionex Ultimate 3000, Thermo Fisher Scientific, Waltham, Ma, USA) coupled to a Q Exactive Orbitrap mass spectrometer (Thermo Fischer Scientific, Waltham, Ma, USA). The UHPLC system consisted of a degassing system, a Quaternary UHPLC pump working at 1250 bar, an auto sampler device and a thermostated Luna Omega column (50 x 2.1 μm, 1.6 μm, Phenomenex) held at 30 °C. The mobile phase consisted of (A) water with 0.1% formic acid containing 5 mM ammonium formate and (B) methanol with 0.1% formic acid containing 5 mM ammonium formate. A linear gradient elution program was applied as follows: initial 0% B was held for 1 min and then increased to 95% B in 1 min and held for 0.5 min. Then, the gradient was linearly decreased to 75% in 2.5 min, and decreased again to 60% B in 1 min. Finally, gradient was reduced to 0% in 0.5 min and held for 1.5 min for re-equilibration, giving a total run time of 8 min. The flow rate was 0.4 mL/min.

Detection was performed using a Q-Exactive Orbitrap mass spectrometer. The mass spectrometer was operated in positive and negative ion mode by setting two scan events: full ion MS and data-dependent fragmentation, dd-MS^2. Full scan data were acquired at a resolving power of 70000 FWHM at 200 *m/z*. Mass range in the Full scan experiments was set at *m/z* 100–1000. The conditions in positive ionization mode (ESI^+) were: spray voltage 4000 V; capillary temperature 290°C; S-lens RF level 50; sheath gas pressure (N_2>95%) 35, auxiliary gas (N_2 >95%) 10, and auxiliary gas heater temperature 305°C. The conditions in negative ionization mode (ESI^-) were: spray voltage - 4000 V; capillary temperature 290°C; S-lens RF level 50;

sheath gas pressure (N_2>95%) 35, auxiliary gas (N_2 >95%) 10, and auxiliary gas heater temperature 305°C. The parameters for the scan event of dd-MS^2 were as follow: a mass resolving power of 17 500 FWHM (*m*/*z* 200), AGC target at 2E5, maximum IT at 50 ms and isolation window at 2.0 *m*/*z*.

A theoretical database including the chemical formula, the extracted mass, the polarity of acquisition and the expected adducts was built and used for post-run retrospective screening (Table 4). To create the database, two spectral libraries were taken: Antibiotics and veterinary drugs Spectral Library v1.0 1.0 for Use in MasterView™ Software, and LibraryView™ Software, AB SCIEX, Mycotoxin Spectral Library for LibraryView™ Software, AB SCIEX. For an accurate mass measurement, the identification and confirmation approach was performed setting a mass tolerance of 5 ppm for the molecular ion and for the fragments; an intensity threshold of 1000.

Data analysis and processing were evaluated by the Quan/Qual Browser Xcalibur software, v. 3.1.66. (Xcalibur, Thermo Fisher Scientific).

Table 4. Chromatographic retention time, optimized MS/MS parameters for untarget analytes.

Compound	Chemical Formula	Extracted Mass (m/z)	Adduct ion
Veterinary drugs: (37)			
Albendazole	$C_{12}H_{15}N_3O_2S$	266.09577	$[M+H]^+$
Betamethasone	$C_{22}H_{29}FO_5$	451.21266	$[M+H_3C_2O_2]^-$
Carprofen	$C_{15}H_{12}ClNO_2$	272.04838	$[M-H]^-$
Cefalexin	$C_{16}H_{17}N_3O_4S$	348.10125	$[M+H]^+$
Cefazolin	$C_{14}H_{14}N_8O_4S_3$	455.03729	$[M+H]^+$
Chlorpromazine	$C_{17}H_{19}ClN_2S$	319.10302	$[M+H]^+$
Chlortetracycline	$C_{22}H_{23}ClN_2O_8$	479.12157	$[M+H]^+$
Cloxacillin	$C_{19}H_{18}ClN_3O_5S$	453.09939	$[M+NH_4]^+$
Cypermethrin	$C_{22}H_{19}Cl_2NO_3$	433.10802	$[M+NH_4]^+$
Decoquinate	$C_{24}H_{35}NO_5$	418.25880	$[M+H]^+$
Diazinon	$C_{12}H_{21}N_2O_3PS$	305.10833	$[M+H]^+$
Dicloxacillin	$C_{19}H_{17}N_3Cl_2O_5S$	470.03387	$[M+H]^+$
Difloxacin	$C_{21}H_{19}F_2N_3O_3$	400.14672	$[M+H]^+$
Dimetridazole	$C_5H_7N_3O_2$	142.06110	$[M+H]^+$
Enrofloxacin	$C_{19}H_{23}FN_3O_3$	360.17179	$[M+H]^+$
Febantel	$C_{20}H_{22}N_4O_6S$	447.13328	$[M+H]^+$
Fenbendazole	$C_{15}H_{13}N_3O_2S$	300.08012	$[M+H]^+$
Florfenicol	$C_{12}H_{14}Cl_2FNO_4S$	355.99318	$[M-H]^-$

Flumequine	$C_{14}H_{12}FNO_3$	262.08739	$[M+H]^+$
Levamisole	$C_{11}H_{12}N_2S$	205.07939	$[M+H]^+$
Lyncomycin	$C_{18}H_{34}N_2O_6S$	407.22103	$[M+H]^+$
Marbofloxacin	$C_{17}H_{19}FN_4O_4$	363.14630	$[M+H]^+$
Mebendazole	$C_{16}H_{13}N_3O_3$	296.10296	$[M+H]^+$
Methylprednisolone	$C_{22}H_{30}O_5$	433.22207	$[M+H_3C_2O_2]^-$
Oxacillin	$C_{19}H_{19}N_3O_5S$	419.13837	$[M+NH_4]^+$
Oxfendazole	$C_{15}H_{13}N_3O_3S$	316.07503	$[M+H]^+$
Oxolinic acid	$C_{13}H_{11}NO_5$	262.07099	$[M+H]^+$
Oxytetracycline	$C_{22}H_{24}N_2O_9$	461.15550	$[M+H]^+$
Prednisolone	$C_{21}H_{28}O_5$	419.20642	$[M+H_3C_2O_2]^-$
Rifaximin	$C_{43}H_{51}N_3O_{11}$	786.35960	$[M+H]^+$
Ronidazole	$C_6H_8N_4O_4$	201.06183	$[M+H]^+$
Spiramycin	$C_{43}H_{74}N_2O_{14}$	843.52128	$[M+H]^+$
Tetracycline	$C_{22}H_{24}N_2O_8$	445.16050	$[M+H]^+$
Thiamphenicol	$C_{12}H_{15}Cl_2NO_5S$	353.99752	$[M-H]^-$
Tilmicosin	$C_{46}H_{80}N_2O_{13}$	869.57331	$[M+H]^+$
Tolfenamic acid	$C_{14}H_{12}ClNO_2$	262.06293	$[M+H]^+$
Toltrazuril	$C_{18}H_{14}F_3N_3O_4S$	424.05843	$[M-H]^-$
Mycotoxins: (16)			
Chaetoglobosin A	$C_{32}H_{36}N_2O_5$	529.26970	$[M+H]^+$
Cyclopiazonic acid	$C_{20}H_{20}N_2O_3$	337.15467	$[M+H]^+$
Deoxynivalenol-3-glucoside	$C_{21}H_{30}O_{11}$	459.18609	$[M+H]^+$
Gliotoxin	$C_{13}H_{14}N_2O_4S_2$	327.04678	$[M+H]^+$
Hydrolyzed fumonisin B_1	$C_{22}H_{47}NO_5$	406.35270	$[M+H]^+$
Hydrolyzed fumonisin B2	$C_{22}H_{47}NO_4$	390.35778	$[M+H]^+$
Penicillic acid	$C_8H_{10}O_4$	171.06518	$[M+H]^+$
Tentoxin	$C_{22}H_{30}N_4O_4$	415.23398	$[M+H]^+$
Verruculogen	$C_{27}H_{33}N_3O_7$	512.23913	$[M+H]^+$
AFM2	$C_{17}H_{14}O_7$	331.08123	$[M+H]^+$
OTA B	$C_{20}H_{19}NO_6$	370.12851	$[M+H]^+$
α-ota	$C_{11}H_9N_5Cl$	247.06192	$[M+H]^+$
Sterigmatocystin	$C_{18}H_{12}O_6$	325.07066	$[M+H]^+$
FB3	$C_{34}H_{59}NO_{14}$	706.40083	$[M+H]^+$
DOM	$C_{15}H_{20}O_5$	281.13835	$[M+H]^+$
T2-trial	$C_{20}H_{30}O_7$	383.20643	$[M+NH_4]^+$

Results and discussion

Optimization of the UHPLC-Q-Orbitrap HRMS conditions

Chromatographic conditions were studied to achieve the best separation of the analyzed compounds. In the present study, methanol was chosen as the organic phase because it achieved a better resolution and sensitivity than acetonitrile for the studied compounds as previously reported in literature (Xie *et al.* 2015).

The addition of formic acid and ammonium formate in mobile phases could promote the formation of adduct ions, thereby improving the detection sensitivity, especially for fumonisins and some drugs, which could hardly ionize under the neutral or alkaline conditions (Mao *et al.* 2018). For instance, the addition reported help in the separation of the Zearalenone isomers without which, these isomers overlapped and could not be quantified separately (Jia *et al.* 2014). In this study, the UHPLC Luna Omega column (50 x 2.1 μm, 1.6 μm, Phenomenex) was used and several gradient profile were tested to optimize the chromatographic separation of the studied compounds. The tested gradients were:

Gradient 1:

The following gradient was tested: initial 10% B was held for 2 min and then increased to 100% B in 3 min and held for 1.5 min. Then, the gradient was linearly decreased to 10% B in 1 min and held for 2 min for re-equilibration, giving a total run time of 9.5 min.

Gradient 2:

The following gradient was tested: initial 5% B was held for 1 min and then increased to 95% B in 1.5 min and held for 1 min and decreased again to 70% B in 2 min. Then, the gradient was linearly decreased to 5% B in 1 min and held for 2 min for re-equilibration, giving a total run time of 9.5 min.

Gradient 3:

The following gradient was tested: initial 0% B was held for 1 min and then increased to 95% B in 1 min and held for 0.5 min. Then, the gradient was linearly decreased to 75% in 2.5 min, and decreased again to 60% B in 1 min. Finally, the gradient was reduced to 0% in 0.5 min and held for 1.5 min for re-equilibration, giving a total run time of 8 min.

Starting the gradient program with 10% B, some of the target analytes peaks were eluted in the column dead time and the peak response was irregular. With the second tested gradient, there was a decrease in the number of analytes no retained from the chromatographic column, but satisfactory results in terms of retention time and good peak shape were achieved using 0% B in the initial condition of the gradient. In the latter, a good separation of all fifty-four target analytes was obtained in 8 min which allowed also a reduction in solvent consumption. Under the condition, retention times of the analytes were constant, ranging from 3.84 (Amoxicillin) to 7.10 (Doramectin) min, as shown in Table 5.

Table 5. Chromatographic retention time, optimized MS/MS parameters for target analytes.

Compound	Retention time (min)	Chemical Formula	Adduct ion	Theoretical mass (m/z)	Measured mass (m/z)	Accuracy (Δ ppm)
Veterinary drug: (24)						
Amoxicillin	3.84	$C_{16}H_{19}N_3O_5S$	$[M+H]^+$	366.11182	366.11173	-0.25
Metromidazole	3.86	$C_6H_9N_3O_3$	$[M+H]^+$	172.07167	172.07152	-0.87
Procaine Benzylpenicillin	4.03	$C_{13}H_{20}N_2O_2$	$[M+H]^+$	237.15975	237.15936	-1.64
Imidocarb	4.10	$C_{19}H_{20}N_6O$	$[M+H]^+$	349.17714	349.17679	-1.00
Danofloxacin	4.15	$C_{19}H_{20}FN_3O_3$	$[M+H]^+$	358.15615	358.15552	-1.76
Dapsone	4.23	$C_{12}H_{12}N_2SO_2$	$[M+H]^+$	249.06922	249.06886	-1.45
Ampicillin	4.27	$C_{16}H_{19}N_3O_4S$	$[M+H]^+$	350.11693	350.11633	-1.63
Sulfadimidine	4.27	$C_{12}H_{14}N_4O_2S$	$[M+H]^+$	279.09102	279.09064	-1.36
Clenbuterol	4.35	$C_{12}H_{18}Cl_2N_2O$	$[M+H]^+$	277.08690	277.08658	-1.15
Trichlorfon	4.40	$C_4H_8Cl_3O_4P$	$[M+H]^+$	256.92985	256.92966	-0.74
Ceftiofur	4.48	$C_{19}H_{17}N_5O_7S_3$	$[M+H]^+$	524.03629	524.03558	-1.35
Colchicine	4.50	$C_{22}H_{25}NO_6$	$[M+H]^+$	400.17546	400.17471	-1.87
Dexamethasone	4.70	$C_{22}H_{29}FO_5$	$[M+H]^+$	393.20718	393.20639	-2.01
Meloxicam	4.79	$C_{14}H_{13}N_3O_4S_2$	$[M-H]^-$	350.02747	350.02710	-1.06
Chloramphenicol	5.11	$C_{11}H_{12}Cl_2N_2O_5$	$[M-H]^-$	321.00505	321.00476	-0.90
Ivermectin	5.60	$C_{48}H_{74}O_{14}$	$[M+NH4]^+$	892.54168	892.54028	-1.57
Amitraz	5.75	$C_{19}H_{23}N_3$	$[M+H]^+$	294.19647	294.09150	-1.36
Cyhalothrin	5.77	$C_{23}H_{19}ClF_3NO_3$	$[M+NH4]^+$	467.13438	467.13361	-1.65
Cypermethrin	5.80	$C_{22}H_{19}Cl_2NO_3$	$[M+NH4]^+$	433.10802	433.10724	-1.80
Deltamethrin	5.87	$C_{22}H_{19}Br_2NO_3$	$[M+NH4]^+$	521.00699	521.00627	-1.38
Eprinomectin	6.07	$C_{50}H_{75}NO_{14}$	$[M+H]^+$	914.52603	914.52417	-2.03
Abamectin	6.15	$C_{48}H_{72}O_{14}$	$[M+NH4]^+$	890.52603	890.52411	-2.16
Monesin	6.50	$C_{36}H_{61}NaO_{11}$	$[M+H]^+$	693.41843	693.41663	-2.60
Doramectin	7.10	$C_{50}H_{74}O_{14}$	$[M+NH4]^+$	916.54168	916.53955	-2.32
Mycotoxins: (30)						
NIV	3.98	$C_{15}H_{20}O_7$	$[M+Na]^+$	335.11012	335.06511	-1.22
DON	4.10	$C_{15}H_{20}O_6$	$[M+H]^+$	297.13326	297.13327	0.03
FUS-X	4.28	$C_{17}H_{22}O_8$	$[M+Na]^+$	37712073	377.12057	-0.42
NEO	4.32	$C_{19}H_{26}O_8$	$[M+NH4]^+$	400,19659	400.19662	0.07

DAS	4.41	$C_{19}H_{26}O_7$	$[M+H]^+$	367.17513	367.17554	1,12
3-ADON	4.45	$C_{17}H_{22}O_7$	$[M+H]^+$	339.14383	339.14331	-1.53
15-ADON	4.48	$C_{17}H_{22}O_7$	$[M+H]^+$	339.14383	339.14331	-1.53
AFM1	4.51	$C_{17}H_{12}O_7$	$[M+H]^+$	329.06558	329.06511	0.09
AFG2	4.52	$C_{17}H_{14}O_7$	$[M+H]^+$	331.08123	331.08032	-2.75
AFG1	4.55	$C_{17}H_{12}O_7$	$[M+H]^+$	329.06558	329.06553	-0,15
AFB2	4.60	$C_{17}H_{14}O_6$	$[M+H]^+$	315.08631	315.08521	-3.49
AFB1	4.64	$C_{17}H_{12}O_6$	$[M+H]^+$	313.07066	313.06958	-3.45
FB1	4.75	$C_{34}H_{59}NO_{15}$	$[M+H]^+$	722.39575	722.39539	-0.50
HT-2	4.77	$C_{22}H_{32}O_8$	$[M+NH4]^+$	442.24354	442.24323	-0.70
α-ZAL	4.81	$C_{18}H_{26}O_5$	$[M-H]^-$	321.17044	321.17136	2.86
AOH	4.85	$C_{14}H_{10}O_5$	$[M-H]^-$	257.04555	257.04623	2.65
α-ZOL	4.87	$C_{18}H_{24}O_5$	$[M-H]^-$	319.15510	319.15561	1.60
T-2	4.88	$C_{24}H_{34}O_9$	$[M+NH4]^+$	484.25411	484.25418	0.14
β-ZAL	4.94	$C_{18}H_{26}O_5$	$[M-H]^-$	321.17044	321.17136	2.86
OTA	4.97	$C_{20}H_{18}NO_6Cl$	$[M+H]^+$	404.08954	404.08801	-3.79
FB2	4.97	$C_{34}H_{59}NO_{14}$	$[M+H]^+$	706.40083	706.40192	1.54
β-ZOL	4.98	$C_{18}H_{24}O_5$	$[M-H]^-$	319.15510	319.15549	1.22
ZAN	5.00	$C_{18}H_{24}O_5$	$[M-H]^-$	319.15510	319.15549	1.22
ZEA	5.02	$C_{18}H_{22}O_5$	$[M+H]^+$	317.13945	317.1391	-1.10
AME	5.14	$C_{15}H_{12}O_5$	$[M-H]^-$	271.06120	271.06131	0.41
ENN B	5.61	$C_{33}H_{57}N_3O_9$	$[M+NH4]^+$	657.44331	657.44299	-0.49
ENN B1	5.73	$C_{34}H_{59}N_3O_9$	$[M+NH4]^+$	671.45986	671.45923	-0.94
BEA	5.77	$C_{45}H_{57}N_3O_9$	$[M+NH4]^+$	801.44331	801.44318	-0.16
ENN A1	5.89	$C_{35}H_{61}N_3O_9$	$[M+NH4]^+$	685.47461	685.47351	-1.60
ENN A	6.07	$C_{36}H_{63}N_3O_9$	$[M+NH4]^+$	699.49026	699.48926	-1.43

The optimum MS/MS parameters for the identification and quantification of the fifty-four analytes were obtained infusing directly each analyte standard (1 μg/mL)into the Q-Orbitrap system using a syringe injection at a flow rate of 8 μL/min in order to obtain all the characteristic molecular ions signals and investigating on operational parameters such as flow gas, temperatures, capillary voltage and collision energy. Sensitivity of target analytes was checked by recording chromatograms in full scan method in both positive and negative ionization mode.

Positive and negative ionization full scan mode were monitored to determine the parameters that lead to the optimal response. The responses of adduct protonate $[M+H]^+$ ions were much more intense than the adduct deprotonate $[M-H]^-$ ions for the studied analytes. After full scan analysis, the accurate mass of the characteristic ion (precursor ion) was listed in a

inclusion list. In dd-MS^2 mode, a product ion spectrum was obtained automatically. With respect to mass analyzer used by others authors like a triple-quadrupole, the chosen mode offers a higher level of confidence in analyte identification. In table 6 are shown the analytical methods for the simultaneous determination of mycotoxins and veterinary drugs reported to date by other authors.

Table 6. Analytical methods for the simultaneous determination of mycotoxins and veterinary drugs reported to date by other authors.

Matrix	n. analytes detected	Extraction/ Clean-up	Separation		Determination		References
			Column	Mobile phase	Detection	Sensitivity (ng/g ng/L)	
Egg	15 veterinary drugs 6 mycotoxins	QuEChERS	LC ACE silica C18 (150 mm×2.1 mm, 3 µm)	A: 0.1% FA in water B: 0.1% FA in acetonitrile Flow:0.2 mL/min	QqQ (API 3000 triple) (ESI) MRM mode	LOQ 1.3-28.9 LOQ *n.r.*	Capriotti *et al.* 2012
Milk Cereal-based products	8 veterinary drugs 12 mycotoxins	QuEChERS	UHPLC BEH C18 (100mm x 2.1mm, 1,7 µm)	A:1 mM NH4OAc and 0.05% AcOH B:0.05% AcOH in methanol Flow: 0.2 mL/min	Q-Orbitrap (Exactive, Thermo Fisher Scientific) (ESI +) and (ESI -) HRMS	LOD 1-100	Dominicis *et al.* 2012
Milk	255 veterinary drugs 6 mycotoxins	Ethanol–acetonitrile (1:5, v/v)- Based	UPLC Acquity HSS-T3 column (100mm x 2.1mm, 1,8 µm)	A: 0.1% FA and 0.5 mmol/LNH4OAc in water B: 0.1% FA in methanol C: 2.5 mmol/LNH4OAc in water D: Methanol Flow:0.4 mL/min	QqQ (Xevo TQ-S) (ESI +) and (ESI -) MRS mode	LOQ 0.05- 5	Zhan *et al.* 2012
Milk Dairy products	27 veterinary drugs 16 mycotoxins	SPE	LC Agilent ZORBAX SB-C18 (100 × 2.1 mm, 3.5 µm)	A: 0.1% FA and 5 mM NH4OAc in water B: Methanol Flow: 0.4 mL/min	QqQ (Agilent 6460) (ESI +) and (ESI -) MRM mode	LOQ 0.02-1.0	Xie *et al.* 2015
Fruits Baby Food	117 veterinary drugs 21 mycotoxins	QuEChERS	UHPLC Eclipse-Plus C18 (50 mm × 2.1 mm, 1.8 µm)	A: 0.1 % FA in water B: 0.1 % FA in acetonitrile Flow: 0.5 mL/min	Q-TOF MS (Agilent 6530) (ESI +) and (ESI -) MRM mode	LOQ 1-100	Pérez-Ortega *et al.* 2016
Vegetable Fruits Cereals Meat Milk	14 veterinary drugs 4 mycotoxins	Acidified methanol-Based	LC ZIC-HILIC-Philic (150 mm × 4.6 mm, 5 µm)	A: 50 mM NH4HCO2 in water B: water/acetonitrile 10/90 (v/v). Flow: 0.5 mL/min	QqQ (Varian model 1200L) (ESI +) MRM mode	LOQ 1-10	Danezis *et al.* 2016
Milk Egg	65 veterinary drugs 39 mycotoxins	QuEChERS	UPLC Acquity CORTECS (3.0 mm × 150 mm, 1.6 µm)	A: 0.1% FA in water B:0.1% FA in acetonitrile Flow: 0.4 mL/min	QqQ (Xevo TQ-S) (ESI +) and (ESI -) MRM mode	LOQ 0.01-31 LOQ 0.01-10	Zhou *et al.* 2017
Milk	24 veterinary drugs 26 mycotoxins	QuEChERS	UHPLC Luna Omega column (50 mm x 2.1 µm, 1.6 µm)	A: : 5 mM NH4HCO2 and 0.1% FA in water B: 5 mM NH4HCO2 and 0.1% FA in methanol Flow: 0.4 mL/min	Q-Orbitrap (Exactive, Thermo Fisher Scientific) (ESI +) and (ESI -) HRMS	LOQ 0.00001-0.03 LOQ 0.001-0.5	This work

References:

- Capriotti, A. L., Cavaliere, C., Piovesana, S., Samperi, R., & Laganà, A. (2012). Multiclass screening method based on solvent extraction and liquid chromatography–tandem mass spectrometry for the determination of antimicrobials and mycotoxins in egg. *Journal of Chromatography A*, *1268*, 84-90.
- Danezis, G. P., Anagnostopoulos, C. J., Liapis, K., & Koupparis, M. A. (2016). Multi-residue analysis of pesticides, plant hormones, veterinary drugs and mycotoxins using HILIC chromatography–MS/MS in various food matrices. *Analytica chimica acta*, *942*, 121-138.
- De Dominicis, E., Commissati, I., & Suman, M. (2012). Targeted screening of pesticides, veterinary drugs and mycotoxins in bakery ingredients and food commodities by liquid chromatography-high-resolution single-stage Orbitrap mass spectrometry. *Journal of Mass Spectrometry*, *47*(9), 1232-1241.
- Jia, W., Chu, X., Ling, Y., Huang, J., & Chang, J. (2014). Multi-mycotoxin analysis in dairy products by liquid chromatography coupled to quadrupole orbitrap mass spectrometry. *Journal of Chromatography A*, *1345*, 107-114.
- Mao, J., Zheng, N., Wen, F., Guo, L., Fu, C., Ouyang, H& Lei, S. (2018). Multi-mycotoxins analysis in raw milk by ultra high performance liquid chromatography coupled to quadrupole orbitrap mass spectrometry. *Food Control*, *84*, 305-311.
- Pérez-Ortega, P., Lara-Ortega, F. J., Gilbert-López, B., Moreno-González, D., García-Reyes, J. F., & Molina-Díaz, A. (2017). Screening of over 600 pesticides, veterinary drugs, food-packaging contaminants, mycotoxins, and other chemicals in food by ultra-high performance liquid chromatography quadrupole time-of-flight mass spectrometry (UHPLC-QTOFMS). *Food Analytical Methods*, *10*(5), 1216-1244.
- Xie, J., Peng, T., Zhu, A., He, J., Chang, Q., Hu, X& Li, J. (2015). Multi-residue analysis of veterinary drugs, pesticides and mycotoxins in dairy products by liquid chromatography–tandem mass spectrometry using low-temperature cleanup and solid phase extraction. *Journal of Chromatography B*, *1002*, 19-29.
- Zhan, J., Yu, X. J., Zhong, Y. Y., Zhang, Z. T., Cui, X. M., Peng, J. F., & Zhu, Y. (2012). Generic and rapid determination of veterinary drug residues and other contaminants in raw milk by ultra performance liquid chromatography–tandem mass spectrometry. *Journal of Chromatography B*, *906*, 48-57.

SPECIFIC OBJECTIVES 2:

Development of a multi-residue method based on QuEChERS procedure for the extraction and purification of veterinary drug residues and mycotoxins in milk.

Materials and Methods

Chemicals and materials

Methanol (MeOH), acetonitrile (AcN) and water(LC-MS grade) were acquired from Merk (Darmstadt, Germany). Formic acid (mass spectrometry grade) was purchased from Fluka (Milan, Italy). Syringe filters with polytetrafluoroethylene membrane (PTFE, 15 mm, diameter 0.22 μm) were supplied by Phenomenex (Castel Maggiore, Italy). Sodium chloride (NaCl), anhydrous sulphate sodium (Na_2SO_4) andanhydrous sodium acetate (NaAc), were acquired from Sigma Aldrich (Milan, Italy). C_{18} (analytical grade) and primary secondary amine (PSA) sorbent were acquired from Supelco (Milan, Italy).

Sampling

A total of 56 commercially available milk samples were randomly purchased between January and February 2018 from different supermarkets located in the Campania region, Southern Italy. Samples were shipped to the laboratory in their original packages and stored at 4°C until analysis. Milk analysis was carried out within two days after arrival of samples.

Sample preparation

A QuEChERS-based procedure developed by Zhou *et al* .2018 was selected as a starting point and then slightly modified. Briefly, 10 mL sample were

introduced into a 50 mL Falcon tube, and 2.5 mL of distillate water and 5 mL of acetonitrile containing 3.35% of formic acid (*v*/*v*) were added. The sample was vortexed for 1 min and sonicated for 15 min (vortexed in every five-minute interval). Then, the tube involved the addition of 4.0 g of Na_2SO_4, 1.2 g of NaCl, and 0.5 g of anhydrous NaAc, which was shaken by hand for 1 min and then centrifuged for 3 min at 4000 rpm and 4°C. The supernatant (3 mL) was collected into a 15 mL Falcon tube containing 300 mg of C18 sorbent, 140 mg of PSA and 1.5 g of Na_2SO_4. Subsequently, the mixture was vortexed for 1 min and centrifuged for 1 min at 1500 rpm and 4°C. The supernatant (2 mL) was transferred into a new glass tube and then dried under gentle nitrogen flow at 45°C. Finally, the residue was reconstituted with 0.5 mL of MeOH:H_2O (70:30, *v*/*v*), filtered through a 0.22 µm filter, and 5 µL were injected into the UHPLC-Q-Orbitrap HRMS instrument.

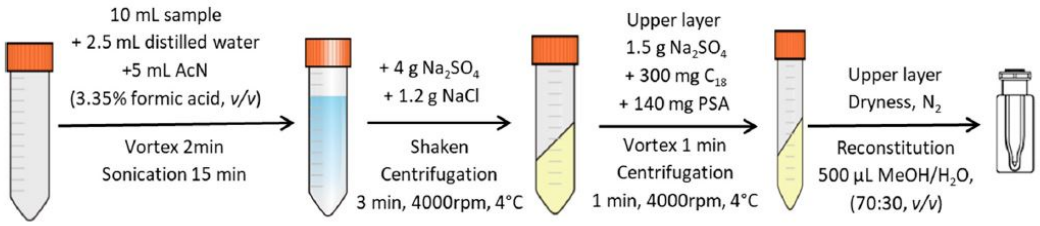


Figure 1. Schematic flow of the sample preparation procedure.

Results and discussion

Optimization of the sample preparation procedure

In the multi-residual determination, sample preparation is a critical step given the different chemical-physical properties of the compounds, the different levels of contaminants to detect and the complexity of the matrix

(Martos *et al.* 2010; Kabir *et al.* 2017). To date, a limited number of information about the simultaneous determination of veterinary drugs and mycotoxins in milk were reported in literature as shown in table 7.

To obtain the optimal experimental conditions different optimization steps were performed. Critical extraction parameters such as different ratios between the amount of sample and the extraction solvent and the ultrasonic treatment were evaluated.

Table 7. Available studies on simultaneous determination of veterinary drugs and micotoxins in milk.

n. analysed milk samples	n. Analytes	% positive samples	Analyte detected	Concentration reported ng/mL	References
20	255 veterinary drugs 6 mycotoxins	25 70	Lincomycin Ciprofloxacin	0.14–13.7 0.59–25.4	Zhan *et al.* 2012
1	8 veterinary drugs 12 mycotoxins	*n.r.*	*n.r.*	*n.r.*	De Dominicis *et al.* 2012
32	27 veterinary drugs 16 mycotoxins	3	Chloramphenicol Flunixin Cimeterol	0.027 0.452 0.053-	Xie *et al.* 2015
n.r.	14 veterinary drugs 4 mycotoxins	*n.r.*	*n.r.*	*n.r.*	Danezis *et al.* 2016
30	65 veterinary drugs 39 mycotoxins	10 43.3 30 36.7	Enoxacin Danofloxacin Ofloxacin Aflatoxin M1	0.5–0.8 0.1–2.7 0.01–1.1 0.002-0.03	Zhou *et al.* 2018
56	61 veterinary drugs 46 mycotoxins	17.5 1.7 1.7 5.3 21 8.8 1.7	Amoxicillin Ampicillin Imidocarb Ivermectin Meloxicam Benzylpenicillin procaine Dexamethasone	1.2-1.68 0.860 0.080 0.380-4.240 0.007-0.220 1-4.530 0.140	This work

The ultrasonic treatment is an important extraction-influencing experimental parameter. In order to improve the extraction efficiency, different ultrasonic times in the extraction process was tested and compared (5, 15 and 25 min). A sonication time of 5 min was not suitable (mean recovery=50%, RSD=22%,*n*=9), whereas mean recoveries of 75% (RSD=12%,

n=9) and 77% (RSD=13%, *n=9*) were obtained using 15 and 25 minutes of ultrasonic treatment, respectively. No significant statistical differences were found between the different time of treatment. Therefore, to keep the sample preparation procedure as short as possible, protocol with 15 minutes of ultrasonic treatment was selected.

After the optimization of ultrasonic treatment, another parameter was streamlined. As regarding the different ratios between the amount of sample and the extraction solvent, three different concentration factors (CF) were tested. Starting to main extraction procedure in which 5 mL of sample was extracted using 10 mL of solvent, a reducing of the solvent volume was performed from 10 to 5 mL (FC 4). To further concentrate, the use of 10 mL of sample (FC 8) leaving unchanged the solvent volume (5 mL) was tested.

Better recoveries (in the range from 52 to 87%) and lower quantification level (LOQ <0.001 ng/L) were obtained with the lower extraction solvent volume tested. With an additional sample concentration (FC8), an important improvement concerning the values of recoveries (from 72 to 93%; RSDs<9%; *n=9*)and a decrease of LOQ (0.0001-0.05 ng/L) were obtained.

All experiments were performed in blank milk samples fortified with studied veterinary drugs and mycotoxins at different levels as reported in the previous section. The analysis was performed in triplicate and the extraction recoveries were expressed as recovery ± RSD (%).

References:

- Kabir, A., Locatelli, M., & Ulusoy, H. I. (2017). Recent trends in microextraction techniques employed in analytical and bioanalytical sample preparation. *Separations*, *4*(4), 36.
- Martos, P. A., Jayasundara, F., Dolbeer, J., Jin, W., Spilsbury, L., Mitchell, M., & Shurmer, B. (2010). Multiclass, multiresidue drug analysis, including aminoglycosides, in animal tissue using liquid chromatography coupled to tandem mass spectrometry. *Journal of agricultural and food chemistry*, *58*(10), 5932-5944.
- Zhou, J., Xu, J. J., Cong, J. M., Cai, Z. X., Zhang, J. S., Wang, J. L., & Ren, Y. P. (2018). Optimization for quick, easy, cheap, effective, rugged and safe extraction of mycotoxins and veterinary drugs by response surface methodology for application to egg and milk. *Journal of Chromatography A*, *1532*, 20-29.

SPECIFIC OBJECTIVES 3

Validation of the analytical method in accordance with the regulation 2002/657/CE on the performance of analytical method.

Materials and methods

Validation of the method

The method was in-house validated according to the guidelines established by the EU Commission Decision 2002/657/EC. The parameters evaluated were: linearity, matrix effect, specificity, trueness, precision and sensitivity. All parameters were performed in triplicate. For confirmation criteria, retention times of analytes in standards and samples were compared at a tolerance of ± 2.5%.

Data quality was monitored using a comprehensive range of Quality Assurance/Quality Control (QA/QC) procedures. A rigorous and systematic control of blanks including: a reagent blank, a procedural blank, a replicate sample, and a matrix-matched external calibration were included in each batch of samples. Three spiking levels for each stock solution were used for analytical quality control (QC).

Statistical data

All method performance experiments were conducted in triplicate and data were expressed as mean ± RSD. The statistical analysis of the results was performed by Student's *t* test. The level of $p \leq 0.05$ was considered statistically significant.

Results and discussion

Analytical features of the proposed method

The proposed method was in-house validated according to the EU Commission Decision 2002/657/EC. Table 8 shows the performance of optimized method. Standard calibration curves and matrix-matched calibration of each analyte were performed at six concentration levels. Each calibration curve was prepared in triplicate. Regression coefficients (R^2) greater than 0.990 were obtained for all studied compounds. To evaluate matrix effects, the matrix-matched calibration curves (A) were compared to the standard calibration curves (B) and expressed as the ratio percentage between these slopes. Thus, the ratio (A/B × 100) was defined as the matrix effect (%). A value of ME 100% means that no matrix effect occurred. There was signal suppression if the value was lower than 100% and signal enhancement if the value was higher than 100%. Matrix effects obtained were in the range of 75-97%. The specificity was determined by analyzing ten times a blank milk extract, to confirm the absence of possible sample interferences at the same retention time of the analyte. For all of the target analytes, the specificity of the HRMS led to no significant interfering substances which could interact with the quantification. To ensure the quality of results, QA/QC criteria were verified. When the recoveries were in the range from 70% and 120% (RSD < 20%) extraction process was considered satisfactory. To evaluate method trueness, recoveries were calculated at different spiking levels. Spiking levels were 0.05, 0.5, 2.5 μg/L for those analytes included in stock 1 solution, 0.005, 0.01, 0.05 μg/L for those analytes included in stock solution 2 and 0.5, 5, 25 μg/L for those analytes included in stock solution 3. The method provided satisfactory recoveries at all spiking

levels, ranging from 74% to 105%. The precision of the method was evaluated in terms of repeatability (intra-day precision, n=3) and reproducibility (inter-day precision, n=3) of milk samples spiked at the levels above reported. The precision data, expressed as percentage relative standard deviation (%RSD), showed that the method was reproducible (RSD<14%) and repeatable (RSD<7%). Sensitivity was determined by calculating the limit of detection (LOD) and limit of quantification (LOQ) for each target compound. LOD was established as the lowest concentration tested that allows to identify the molecular ion with a mass error below 5 ppm. LOQ was established as the lowest concentration of the analyte that generate a chromatographic peak with acceptable accuracy and precision results(<20%).

Table 6 are shown the available multi-residue methods for the simultaneous determination of mycotoxins and veterinary drugs reported by other authors in the last decade. Even though the analysis of contaminants is becoming more attractive for food safety purposes in food, the presence of methods which include compounds from a high variety of different chemical classes is still scarce. A comparison of the analytical methods demonstrated that the proposed method has the higher sensitivity and thus allowed to detect lower levels of analytes. In particular, the value of LOQ achieved with the proposed method allowed to quantify levels ten times lower for mycotoxins and twenty times lower for veterinary drugs compared to available methods in literature. The optimized method, compared to methods used in previous studies turns out to be more sensitive, quick and efficient.

Table 8. Method performance.

Analyte	Linearity (r^2)	SSE (%)	Recovery ± RSD (%, *n*=9)			LOQ (pg/mL)
			0.5 ng/mL	5 ng/mL	25 ng/mL	
Abamectin	0.9966	93	80 ± 9	96 ± 3	101 ± 5	30.00
Amitraz	0.9936	76	80 ± 4	86 ± 6	91 ± 6	30.00
Amoxicillin	0.9937	88	86 ± 5	92 ± 5	96 ± 5	15.00
Ampicillin	0.9986	84	79 ± 6	84 ± 7	87 ± 4	1.00
Ceftiofur	0.9995	95	87 ± 6	95 ± 2	98 ± 3	30.00
Cyhalothrin	0.9903	97	80 ± 9	87 ± 6	92 ± 6	30.00
Cypermethrin	0.9966	75	74 ± 9	80 ± 4	85 ± 4	5.00
Danofloxacin	0.9978	94	84 ± 10	86 ± 4	88 ± 7	0.5
Deltamethrin	0.9968	86	81 ± 8	91 ± 3	94 ± 3	5.00
Dexamethasone	0.9993	94	91 ± 10	96 ± 4	105 ± 3	30.00
Doramectin	0.9993	89	87 ± 8	91 ± 4	97 ± 2	30.00
Eprinomectin	0.9968	98	84 ± 9	92 ± 6	98 ± 6	5.00
Imidocarb	0.9982	82	90 ± 8	95 ± 9	97 ± 8	1.00
Ivermectine	0.9987	97	89 ± 7	95 ± 5	105 ± 4	0.05
Meloxicam	0.9955	90	90 ± 7	94 ± 6	101 ± 5	5.00
Monesin	0.9981	98	88 ± 11	93 ± 5	95 ± 3	30.00
Procaine Benzilpenicillin	0.9974	75	84 ± 6	93 ± 5	99 ± 4	0.05
Sulfadimidine	0.9906	93	86 ± 9	95 ± 7	99 ± 6	1.00
Trichlorfon	0.9981	85	77 ± 14	83 ± 5	87 ± 5	5.00

Analyte	Linearity (r^2)	SSE (%)	Recovery ± RSD (%, *n*=9)			LOQ (pg/mL)
			0.05 ng/mL	0.5 ng/mL	2.5 ng/mL	
DON	0.9985	94	94 ± 4	87 ± 10	83 ± 11	25.00
FUS-X	0.9945	89	96 ± 8	89 ± 9	80 ± 8	25.00
NEO	0.9935	86	92 ± 7	90 ± 8	88 ± 9	25.00
DAS	0.9957	84	97 ± 5	94 ± 11	85 ± 13	50.00
3-ADON	0.9985	90	88 ± 7	87 ± 9	75 ± 15	50.00
15-ADON	0.9901	94	84 ± 6	94 ± 4	88 ± 6	50.00
FB1	0.9926	97	91 ± 3	84 ± 4	80 ± 10	12.50
HT-2	0.9973	91	95 ± 5	91 ± 7	83 ± 6	12.50
α-ZAL	0.9992	81	86 ± 6	84 ± 9	95 ± 11	25.00
AOH	0.9908	85	90 ± 8	84 ± 10	77 ± 12	50.00
α-ZOL	0.9946	86	91 ± 7	93 ± 12	80 ± 14	25.00
T-2	0.9984	87	87 ± 9	90 ± 10	93 ± 7	12.50
β-ZAL	0.9937	94	82 ± 5	85 ± 8	84 ± 9	25.00
OTA	0.9910	93	96 ± 8	93 ± 7	92 ± 10	2.50
FB2	0.9909	97	93 ± 9	90 ± 8	89 ± 6	12.50
β-ZOL	0.9997	85	98 ± 4	84 ± 9	77 ± 8	25.00
ZAN	0.9932	89	88 ± 5	82 ± 7	74 ± 9	25.00
ZEA	0.9974	87	84 ± 3	94 ± 7	84 ± 12	25.00
AME	0.9937	94	89 ± 8	93 ± 6	87 ± 11	50.00
ENN B	0.9954	86	96 ± 6	95 ± 6	91 ± 9	12.50
ENN B1	0.9967	97	94 ± 7	87 ± 4	92 ± 6	12.50
BEA	0.9909	86	84 ± 8	89 ± 8	82 ± 7	25.00
ENN A1	0.9949	97	96 ± 9	93± 8	88 ± 11	12,50
ENN A	0.9943	95	93 ± 4	91± 9	86 ± 13	12,50

Analyte	Linearity (r^2)	SSE (%)	Recovery ± RSD (%, *n*=9)			LOQ (pg/mL)
			0.005 ng/mL	0.01 ng/mL	0.05 ng/mL	
AFG2	0.9934	96	94 ± 6	93 ± 9	90 ± 10	4.00
AFG1	0.9912	89	86 ± 7	89 ± 8	95 ± 12	2.00
AFM1	0.9953	87	93 ± 5	97 ± 7	89 ± 8	2.00
AFB2	0.9940	95	88 ± 7	100 ± 6	91 ± 9	4.00
AFB1	0.9961	83	86 ± 7	96 ± 8	84 ± 8	2.00
Clenbuterol	0.9948	73	98 ± 3	91 ± 4	84 ± 8	0.01
Chloramphenicol	0.9950	82	104 ± 2	96 ± 6	81 ± 9	2.00
Colchicine	0.9957	93	96 ± 2	91 ± 5	83 ± 11	7.5
Dapsone	0.9981	86	88 ± 4	84 ± 8	76 ± 7	0.05
Metronidazole	0.9996	81	94 ± 3	89 ± 5	82 ± 4	1.00

References:

- European Commission Decision (2002/657/EC) of 12 August 2002 Implementing Council Directive 96/23/EC Concerning the Performance of Analytical Methods and Interpretation of Results. 2002. Available online: https://publications.europa.eu/en/publication-detail/-/publication/ed928116-a955- 4a84-b10a-cf7a82bad858/language-en (accessed on 4 October 2018).

SPECIFIC OBJECTIVES 4:

Application of validated method to investigate the occurrence of the before mentioned compounds in 56 milk samples purchased in Campania, Italy.

Results and discussion

Application of the method to milk samples

The validated method was applied to the analysis of 56 milk samples acquired from different supermarkets located in the Campania region, Southern Italy. Results obtained were listed in Table 9.

Table 9. Results obtained applied the validated methods to the 56 milk samples (target analysis).

Milk samples (ng/mL)	Amoxicillin	Ampicillin	Imidocarb	Ivermectin	Meloxicam	Procaine Benzylpenicillin	Dexamethasone
1	1.600	*n.r.*	*n.r.*	*n.r.*	*n.r.*	*n.r.*	*n.r.*
2	*n.r.*	*n.r.*	*n.r.*	*n.r.*	1.540	*n.r.*	*n.r.*
3	*n.r.*	*n.r.*	*n.r.*	1.600	*n.r.*	*n.r.*	*n.r.*
4	1.540	*n.r.*	*n.r.*	*n.r.*	*n.r.*	*n.r.*	*n.r.*
5	*n.r.*	*n.r.*	*n.r.*	*n.r.*	0.015	*n.r.*	*n.r.*
6	*n.r.*	*n.r.*	*n.r.*	*n.r.*	0.018	*n.r.*	*n.r.*
7	*n.r.*	*n.r.*	*n.r.*	*n.r.*	0.012	*n.r.*	*n.r.*
6	*n.r.*	*n.r.*	*n.r.*	*n.r.*	0.008	*n.r.*	*n.r.*
10	*n.r.*	*n.r.*	*n.r.*	0.380	*n.r.*	*n.r.*	*n.r.*
11	1.200	*n.r.*	*n.r.*	*n.r.*	*n.r.*	*n.r.*	*n.r.*
12	1.640	*n.r.*	*n.r.*	*n.r.*	*n.r.*	*n.r.*	*n.r.*
15	*n.r.*	*n.r.*	*n.r.*	*n.r.*	0.014	*n.r.*	*n.r.*
19	*n.r.*	*n.r.*	*n.r.*	*n.r.*	0.008	*n.r.*	*n.r.*
23	*n.r.*	0.860	*n.r.*	*n.r.*	0.010	*n.r.*	*n.r.*
25	1.660	*n.r.*	*n.r.*	*n.r.*	*n.r.*	*n.r.*	*n.r.*
28	*n.r.*	*n.r.*	0.080	*n.r.*	*n.r.*	*n.r.*	*n.r.*
29	*n.r.*	*n.r.*	*n.r.*	*n.r.*	*n.r.*	1.020	*n.r.*
33	*n.r.*	*n.r.*	*n.r.*	*n.r.*	0.020	*n.r.*	*n.r.*
36	1.680	*n.r.*	*n.r.*	*n.r.*	0.060	*n.r.*	*n.r.*
38	*n.r.*	*n.r.*	*n.r.*	*n.r.*	*n.r.*	1.000	*n.r.*
43	*n.r.*	*n.r.*	*n.r.*	*n.r.*	*n.r.*	3.365	*n.r.*
45	*n.r.*	*n.r.*	*n.r.*	*n.r.*	*n.r.*	4.530	*n.r.*
46	1.450	*n.r.*	*n.r.*	*n.r.*	*n.r.*	*n.r.*	*n.r.*
48	1.500	*n.r.*	*n.r.*	*n.r.*	*n.r.*	*n.r.*	*n.r.*
49	*n.r.*	*n.r.*	0.007	*n.r.*	*n.r.*	*n.r.*	*n.r.*
50	1.480	*n.r.*	*n.r.*	*n.r.*	*n.r.*	*n.r.*	*n.r.*
51	*n.r.*	*n.r.*	*n.r.*	4.240	0.220	1.000	*n.r.*
55	1.510	*n.r.*	*n.r.*	*n.r.*	*n.r.*	*n.r.*	*n.r.*

None of the analyzed samples were contaminated by mycotoxins. As regarding the occurrence of mycotoxins in milk, most of the studies reported in the literature are focused on AFM1 investigation. The here reported data, are in agreement with previous monitoring studies carried out in milk. For instance, Tsiplakou *et al.* did not detect AFM1 (LOQ: 0.002 μg/L) in any of Greek milk analyzed samples *(n=21*). Similarly, Flores-Flores *et al.* 2018, who conducted a monitoring of AFM1 in Spanish milk samples *(n=191*) did not found AFM1 contamination (LOD: 0.025 ng/g). Boudra *et al.* have detected AFM1 contamination in 3.4% of French milk samples analyzed *(n=264*) at levels below the EU Regulation. In contrast, Duarte *et al.* 2013 reported that 5% of Croatian milk samples *(n=40*), presented AFM1 values that surpassed the legal maximum limit (0.05 μg/L). As regards the incidence of AFB1 in milk, may be due to contaminated feedstuffs not completely metabolized by cow to AFM1, the occurrence of AFB1 was reported in 1.2% (*n=80*) [Carvajal *et al.* 2003], 1.4% (*n=290*) [Nassib *et al.* 2005], 12.4% (n=250) [Mao *et al.* 2018] and 17.5% (*n=40*) [Scaglioni *et al.* 2014] of Mexican, Egyptian, Chinese, and Brazilian milk samples, respectively. In agreement with previous studies reported in the literature, the absence of AFB1 was found in the here analyzed milk samples (Flores-Flores *et al.* 2017; Flores-Flores *et al.* 2018, Rodriguez *et al.* 2018). As reported in table 7, among the authors that examined the simuntaneous occurrence of mycotoxins and veterinary drug residues in milk, the presence of AFM1 was reported by just Zhou *et al.* 2018.

Even though aflatoxins (especially AFM1) are the mycotoxins of greater incidence in milk and dairy products, have been reported that other mycotoxins, such as fumonisin, ochratoxin A, trichothecenes, zearalenone, T-2 toxin, and deoxynivalenol, can also be found in these products (Flores-Flores *et al.* 2015).

As far as OTA, Pattono *et al.* 2011 reported the occurrence of OTA in 3 Italian milk samples *(n=63)* in concentrations ranging from 0.07 to 0.11 μg/L (LOQ 0.05 μg/L). Also, Boudra *et al.* 2007 reported the occurrence of OTA in 2.3% of French milk samples *(n=132)* above the LOQ of the method (0.005 μg/L). On the contrary, Flores *et al.* 2018 did not found OTA in 191 milk samples (LOD 0.2 μg/L). Over the past decade, attention to the risk to human health has also been extended at *Fusarium* mycotoxins (Gruber *et al.* 2016). Notwithstanding, no presence of *Fusarium* mycotoxins (fumonisins, ZEN, trichothecenes) were found in herein reported data, Gazzotti *et al.* 2009 have found FB1 above the LOQ in eight out of ten analysed Italian samples at a contamination range from 0.26 to 0.38 μg/kg. Furthermore, our results regarding the incidence of ZEN are in agreement with the results reported by Zhan *et al.* 2012 who analyzed seven mycotoxins without detecting samples contamination. Even though ZEN is not one of the higher mycotoxins of occurrence in milk and its derivatives, some studies have reported ZEN contamination (Meucci *et al.* 2011, Signorini *at al.* 2012, Hang *et al.* 2014). Regarding trichothecenes, although have been reported the possibility of milk contaminated toward animals ingesting feed contaminated (Danicke and Brezina, 2013), no available studies reported this occurrence in milk. In fact, Flores *et al.* 2018 who analyzed 22 mycotoxins including DON, 3-DON, 15-ADON, HT2 and T2 did not detected contamination of samples (LOD: 0.4-2.5 μg/L).

Concerning the occurrence of veterinary drugs residues, the contamination was detected in 50% of analyzed samples ata concentration levels from 0.007 ng/mL to 4.53 ng/mL. Among the studied VDs, up to seven analytes, belonging to antibiotic drugs (amoxicillin, ampicillin, benzylpenicillin

procaine), NSAIDs (meloxicam), corticosteroids (dexamethasone), antiprotozoans (imidocarb) andanthelmintics (ivermectina) were detected.

Amoxicillin was found in ten milk samples (17.8%) in a concentration range from 1.200 to 1.680 ng/mL (LOQ: 0.015). Ampicillin was detected in one of the analyzed samples *(n=56)* at a concentration of 0.860 ng/mL (LOQ: 0.001). None of the analyzed veterinary drug residues had a concentration level higher than their permitted limits, except for benzylpenicillin procaine, which was detected in 5 out of 56 analyzed samples (8.9%) in a concentration range from 1.000 to 4.530 ng/mL (EU 4 ng/mL). The occurrence of amoxicillin was reported in 3% of Chinese milk samples *(n*=33) analyzed by Zhu *et al.* 2016 at an average concentration of 22.9 ng/mL, the latter percentage is out of MRL permitted by the legislation in force (EC: 4 ng/mL; China: 10 ng/mL). As shown in table 7, no occurrence of β-Lactams residues were found in milk, whereas, the occurrence of ciprofloxacin (quinolones) and lincomycin (lincosamides) were reported by Zhan *et al.* 2012 in 70% and 25 % of analyzed samples, respectively. Also the incidence of ampicillin did not detect in twenty milk samples (LOQ 0.5 ng/g) analysed by Li *et al.* 2018, and 108 milk samples (LOQ <10 μg/L) analyzed by Stolker *et al.* 2008.

Among the anti-inflammatory veterinary drugs, meloxicam (NSAIDs) was the most commonly detected (21%, *n=12*) in the analyzed milk samples in a concentration range of 0.007-0.220 ng/mL. As regards dexamethasone (glucocorticoids), the occurrence was found in one of the analyzed samples *(n=56)* at a concentration of 0.140 ng/mL (LOQ: 0.030 ng/mL). Despite the prevalence, the levels detected were lower than the MRLs provided by EC Regulation no. 37/2010 in all cases (15 μg/Kg and 0.3 μg/Kg for meloxicam and dexamethasone, respectively). Also, Zhan *et al.* 2012 have investigated anti-inflammatory veterinary drug residues *(n=46)* in twenty milk

samplesbut did not report the occurrence of this classes of drugs (LOQ 0.2-1 μg/kg). Similarly, Xie *et al.* 2015 did not found NSAIDs drugs in analysed *(n=32)* milk samples (LOQ 0.12-1 μg/kg). The absence of dexamethasone in *(n=20)* milk samples was reported also by Li *et al.* 2018.

No presence of prohibited veterinary drug residues in milk such as, chloramphenicol, dapsone, metronidazole, colchicine was reported although the low detection level (LOQ: 0.00001μg/L). According to our results, Stolker *et al.* 2008, did not find the occurrence of prohibited drugs in *(n*=100) Dutch analyzed milk samples (LOQ <10 μg/L). Similarly, Alcántara-Durán *et al.* 2018, who conducted a study of the incidence of *(n*=87) VDs in four Spanish milk samples did not found prohibited drugs (LOD: 0.1-1 μg/kg). On the contrary, as shown in table 7, the presence of chloramphenicol was found in 3% of Chinese milk sample *(n*=32) by Xie *et al.* 2015. In the same study, the absence of metronidazole (LOQ: 0.2 μg/kg) and dapsone (LOQ: 0.04 μg/kg) were reported. Furthermore, Bilandžić *et al.* 2011 detected the occurrence of chloramphenicol in 63.6% of Croatian milk sample (*n=1259*), at a concentration range from 0.001 to 0.118 ng/mL.

Of special toxicological interest is the occurrence of veterinary drug residues in the same sample. In 4 out of 56 milk analyzed samples in this study, the coexistence of veterinary drug residues was detected: three samples contained simultaneously two analytes and one sample three analytes. In the latter, the simultaneous presence of: ivermectin (4.240 ng/mL), meloxicam (0.220 ng/mL) and benzylpenicillin procaine (1 ng/mL) was detected. Even though the individual concentrations of the veterinary drug residues detected in the samples were within the MRL, the possible toxic effects derived from the additive or synergistic action of different

chemical substances coexisting in the same sample should be taken into consideration in the risk assessment studies.

Retrospective screening analysisof real samples

The post-target screening approach has enabled us to detect 26 out of 37 veterinary drugs and 12 out of 16 mycotoxins in 56 real samples, reprocessing the raw data. The results of the retrospective analysis of data are shown in the Figure 2. As regards the incidence of mycotoxins, AFM2 contamination, was displayed in >70% of analyzed samples. Also Lee *et al*. 2015 have studied AFM2 contamination in dairy products reporting a percentage of contamination in the range between 2-10% of the fifty analyzed samples. After ingestion, AFB1 and AFB2 are metabolized by the liver to their hydroxylated metabolites M1 (AFM1) and M2 (AFM2), which can be excreted in urine, feces, transferred to milk. In a recent study conducted by Tozzi *et al.* 2016 individual milk samples were analyzed for AFM1 and AFM2 contamination for 15 days after the contaminated feed administration. Amounts of AFM1 and AFM2 were detected in the analysed milk samples. Concerning the other eleven mycotoxins investigated, a low incidence (<35%) were found.

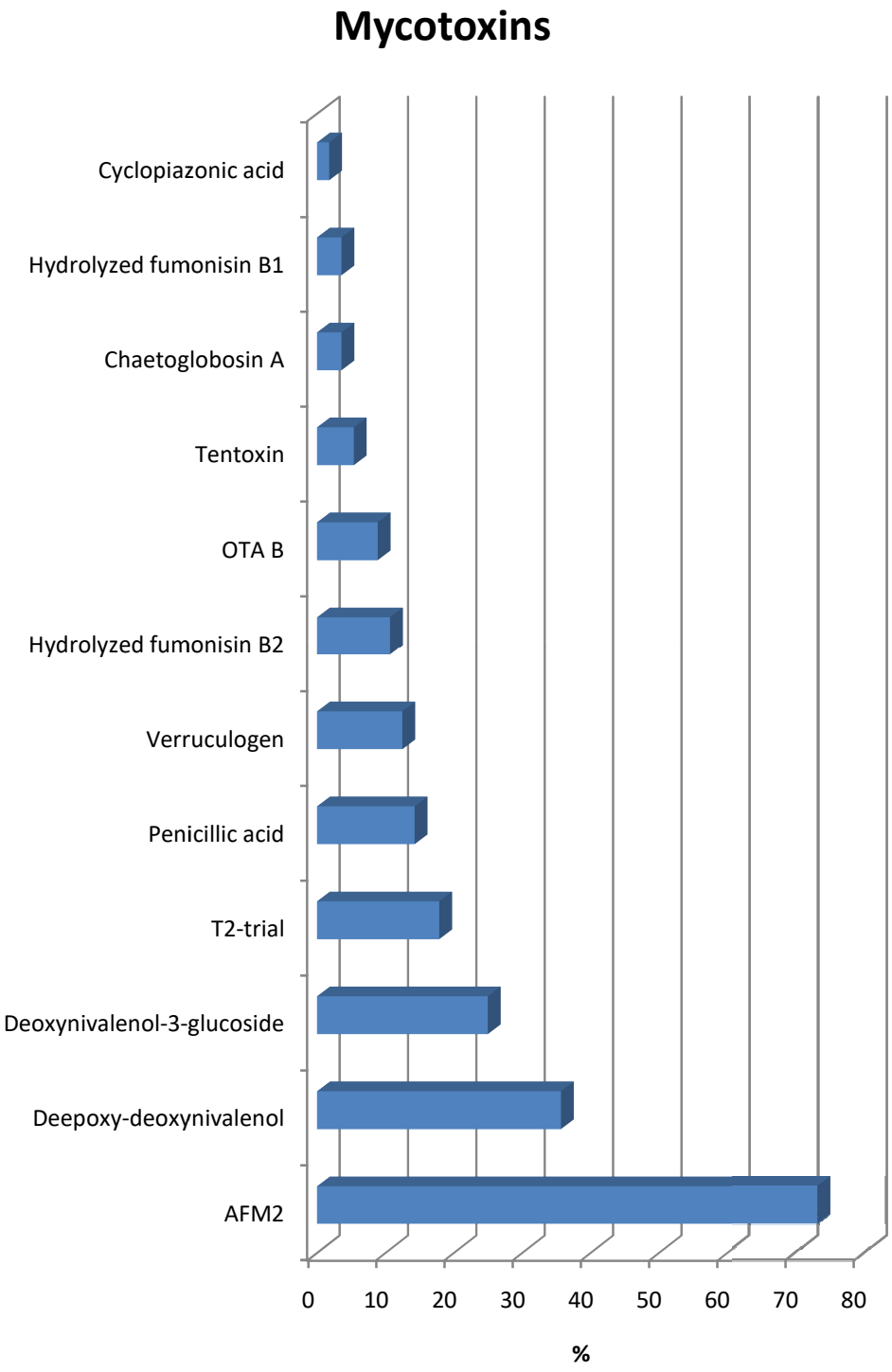
The main part of the texted samples shown a VDs contamination (>40%) by drugs frequently use in food producing animals, like antibiotics and anti-inflammatories presuming an inappropriate use of drugs or no respect of withdrawal period (Masiá *et al.* 2016). A significant percentage (>80%) have shown by betamethasone, prednisolone and oxfendazole. Special focus should be paid to these latter, considering that the maximum residue limit permitted in EC 37/2010 is really low, 0.3; 6; 10 ug/Kg, respectively. In addition, others veterinary drug residues identified in analyzed milk

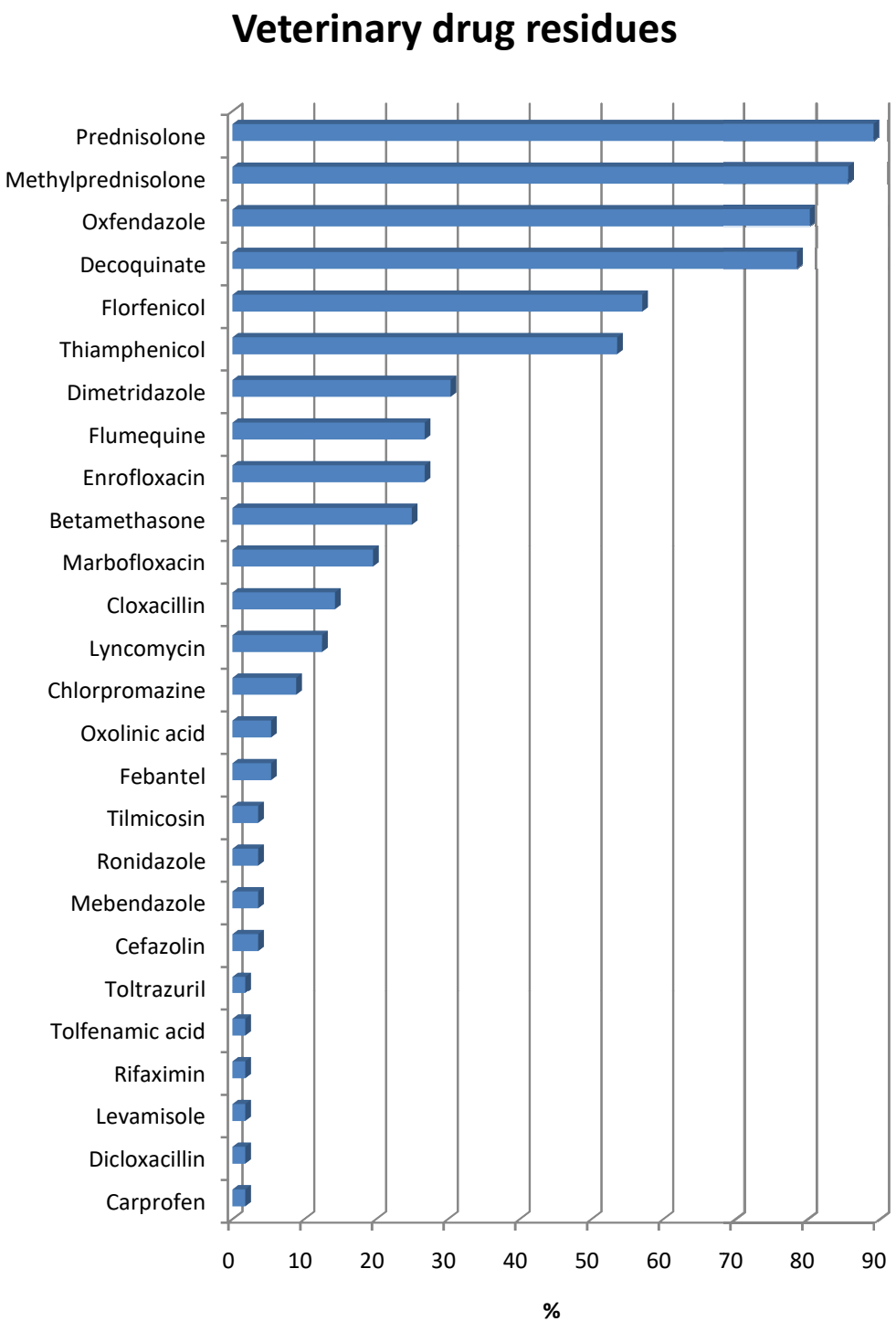
samples are prohibited in milk in accordance with regulation in force (EC 37/2010). Table 10 shown the maximum limits permitted by regulation in force for VDs investigate with a post target analysis.

Table 10. In table the maximum limits permitted by regulation in force for VDs investigate with a post target analysis were reported.

Veterinary drug residues	µg/kg permitted by regulation in force
Betamethasone	0.3
Carprofen	Prohibited in milk
Cefazolin	50
Chlorpromazine	Prohibited in milk
Cloxacillin	30
Decoquinate	Prohibited in milk
Dicloxacillin	30
Dimetridazole	Prohibited in milk
Enrofloxacin	100
Febantel	10
Florfenicol	Prohibited in milk
Flumequine	50
Levamisole	Prohibited in milk
Lyncomycin	150
Marbofloxacin	75
Mebendazole	Prohibited in milk
Methylprednisolone	Prohibited in milk
Oxfendazole	10
Oxolinic acid	Prohibited in milk
Prednisolone	6
Rifaximin	60
Ronidazole	Prohibited in milk
Thiamphenicol	50
Tilmicosin	50
Tolfenamic acid	50
Toltrazuril	Prohibited in milk

Figure 2. Retrospective screening analysis of real samples (untarget analysis).




Veterinary drug residues
Prednisolone
Methylprednisolone
Oxfendazole
Decoquinate
Florfenicol
Thiamphenicol
Dimetridazole
Flumequine
Enrofloxacin
Betamethasone
Marbofloxacin
Cloxacillin
Lyncomycin
Chlorpromazine
Oxolinic acid
Febantel
Tilmicosin
Ronidazole
Mebendazole
Cefazolin
Toltrazuril
Tolfenamic acid
Rifaximin
Levamisole
Dicloxacillin
Carprofen
0
10
20
30
40
50
60
70
80
90
%

References:

- Alcántara-Durán, J., Moreno-González, D., Gilbert-López, B., Molina-Díaz, A., & García-Reyes, J. F. (2018). Matrix-effect free multi-residue analysis of veterinary drugs in food samples of animal origin by nanoflow liquid chromatography high resolution mass spectrometry. *Food chemistry*, *245*, 29-38.
- Bilandžić, N., Varenina, I., & Solomun, B. (2010). Aflatoxin M1 in raw milk in Croatia. *Food Control*, *21*(9), 1279-1281.
- Boudra, H., Barnouin, J., Dragacci, S., & Morgavi, D. P. (2007). Aflatoxin M1 and ochratoxin A in raw bulk milk from French dairy herds. *Journal of Dairy Science*, *90*(7), 3197-3201.
- Carvajal, M., Rojo, F., Méndez, I., & Bolaños, A. (2003). Aflatoxin B1 and its interconverting metabolite aflatoxicol in milk: the situation in Mexico. *Food additives and contaminants*, *20*(11), 1077-1086.
- Dänicke, S., & Brezina, U. (2013). Kinetics and metabolism of the *Fusarium* toxin deoxynivalenol in farm animals: Consequences for diagnosis of exposure and intoxication and carry over. *Food and chemical toxicology*, *60*, 58-75.
- Duarte, S. C., Almeida, A. M., Teixeira, A. S., Pereira, A. L., Falcão, A. C., Pena, A., & Lino, C. M. (2013). Aflatoxin M1 in marketed milk in Portugal: Assessment of human and animal exposure. *Food Control*, *30*(2), 411-417.
- Flores-Flores, M. E., & González-Peñas, E. (2017). An LC–MS/MS method for multi-mycotoxin quantification in cow milk. *Food chemistry*, *218*, 378-385.
- Flores-Flores, M. E., & González-Peñas, E. (2018). Analysis of mycotoxins in Spanish milk. *Journal of dairy science*, *101*(1), 113-117.
- Flores-Flores, M.E.; Lizarraga, E.; de Cerain, A.L.; González-Peñas (2015). E. Presence of mycotoxins in animal milk: A review. *Food Control*,53, 163–176.
- Gruber-Dorninger, C., Novak, B., Nagl, V., & Berthiller, F. (2016). Emerging mycotoxins: Beyond traditionally determined food contaminants. *Journal of agricultural and food chemistry*, *65*(33), 7052-7070.
- Huang, L. C., Zheng, N., Zheng, B. Q., Wen, F., Cheng, J. B., Han, R. W., & Wang, J. Q. (2014). Simultaneous determination of aflatoxin M1, ochratoxin A, zearalenone and α-zearalenol in milk by UHPLC–MS/MS. *Food chemistry*, *146*, 242-249.

- Lee, D., & Lee, K. G. (2015). Analysis of aflatoxin M1 and M2 in commercial dairy products using high-performance liquid chromatography with a fluorescence detector. *Food Control*, 50, 467-471.
- Li, J., Ren, X., Diao, Y., Chen, Y., Wang, Q., Jin, W., & Liu, H. (2018). Multiclass analysis of 25 veterinary drugs in milk by ultra-high performance liquid chromatography-tandem mass spectrometry. *Food chemistry*, *257*, 259-264.
- Mao, J., Zheng, N., Wen, F., Guo, L., Fu, C., Ouyang, H., & Lei, S. (2018). Multi-mycotoxins analysis in raw milk by ultra high performance liquid chromatography coupled to quadrupole orbitrap mass spectrometry. *Food Control*, *84*, 305-311.
- Masiá, A., Suarez-Varela, M. M., Llopis-Gonzalez, A., & Pico, Y. (2016). Determination of pesticides and veterinary drug residues in food by liquid chromatography-mass spectrometry: a review. *Analytica chimica acta*, *936*, 40-61.
- Meucci, V., Razzuoli, E., Soldani, G., & Massart, F. (2010). Mycotoxin detection in infant formula milks in Italy. *Food Additives and Contaminants*, *27*(1), 64-71.
- Nassib, T. A., Guergues, S. N., & Motawee, M. M. (2005). Survey For Detection and Determination of Aflatoxins M1 and B1 in local Milk and Certain Dairy Products by Thin Layer Chromatographic Method. *Egyptian Journal of Hospital Medicine*, *18*, 29-36.
- Pattono, D., Gallo, P. F., & Civera, T. (2011). Detection and quantification of Ochratoxin A in milk produced in organic farms. *Food Chemistry*, *127*(1), 374-377.
- Rodríguez-Carrasco, Y., Izzo, L., Gaspari, A., Graziani, G., Mañes, J., & Ritieni, A. (2018). Simultaneous Determination of AFB1 and AFM1 in Milk Samples by Ultra High Performance Liquid Chromatography Coupled to Quadrupole Orbitrap Mass Spectrometry. *Beverages*, *4*(2), 43.
- Scaglioni, P. T., Becker-Algeri, T., Drunkler, D., & Badiale-Furlong, E. (2014). Aflatoxin B1 and M1 in milk. *Analytica chimica acta*, *829*, 68-74.
- Signorini, M. L., Gaggiotti, M., Molineri, A., Chiericatti, C. A., De Basílico, M. Z., Basílico, J. C., & Pisani, M. (2012). Exposure assessment of mycotoxins in cow's milk in Argentina. *Food and chemical toxicology*, *50*(2), 250-257.
- Stolker, A. A. M., Rutgers, P., Oosterink, E., Lasaroms, J. J. P., Peters, R. J. B., Van Rhijn, J. A., & Nielen, M. W. F. (2008). Comprehensive screening and quantification of veterinary drugs in milk using UPLC–ToF-MS. *Analytical and Bioanalytical Chemistry*, *391*(6), 2309-2322.

- Tozzi, B., Liponi, G. B., Meucci, V., Casini, L., Dall'Asta, C., Intorre, L., & Gatta, D. (2016). Aflatoxins M1 and M2 in the milk of donkeys fed with naturally contaminated diet. *Dairy Science & Technology*, 96(4), 513-523.
- Tsiplakou, E., Anagnostopoulos, C., Liapis, K., Haroutounian, S. A., & Zervas, G. (2014). Determination of mycotoxins in feedstuffs and ruminant' s milk using an easy and simple LC–MS/MS multiresidue method. *Talanta*, *130*, 8-19.
- Xie, J., Peng, T., Zhu, A., He, J., Chang, Q., Hu, X., & Li, J. (2015). Multi-residue analysis of veterinary drugs, pesticides and mycotoxins in dairy products by liquid chromatography–tandem mass spectrometry using low-temperature cleanup and solid phase extraction. *Journal of Chromatography B*, *1002*, 19-29.
- Zhan, J., Yu, X. J., Zhong, Y. Y., Zhang, Z. T., Cui, X. M., Peng, J. F., & Zhu, Y. (2012). Generic and rapid determination of veterinary drug residues and other contaminants in raw milk by ultra performance liquid chromatography–tandem mass spectrometry. *Journal of Chromatography B*, *906*, 48-57.
- Zhou, J., Xu, J. J., Cong, J. M., Cai, Z. X., Zhang, J. S., Wang, J. L., & Ren, Y. P. (2018). Optimization for quick, easy, cheap, effective, rugged and safe extraction of mycotoxins and veterinary drugs by response surface methodology for application to egg and milk. *Journal of Chromatography A*, *1532*, 20-29.
- Zhu, W. X., Yang, J. Z., Wang, Z. X., Wang, C. J., Liu, Y. F., & Zhang, L. (2016). Rapid determination of 88 veterinary drug residues in milk using automated TurborFlow online clean-up mode coupled to liquid chromatography-tandem mass spectrometry. *Talanta*, *148*, 401-411.

3. CONCLUSIONS

A multi-residue method was optimized for the simultaneous determination of veterinary drug residues and mycotoxins in milk. The method has been validated according to Decision 2002/657/CE. In terms of linearity, matrix effect, specificity, trueness, precision and sensitivity, satisfactory characteristics of analytical method has been obtained. The method developed herein, compared to methods used in previous studies turns out to be more sensitive, quick and efficient. The validated UHPLC-Q-Orbitrap HRMS methodology was applied to 56 milk samples commercially available in Italy. Results showed that any analyzed sample was contaminated with mycotoxins whereas 50% of the samples were positive in the presence of veterinary drugs at a concentration range from 0.007 to 4.530 ng/mL Meloxicam was the most commonly detected drugs (21%). Of particular toxicological interest is the co-occurrance of veterinary drug residues in the same milk sample. In herein data reported, co-occurrance of up to three veterinary drug residues was found in four analyzed samples. In addition, retrospective screening analysis of real samples have demostated an high incidence of prohibited VDs in milk (37/2010) and AFM2 contamination in >70% of the analysed samples. Thus, the latter results obtained show the need for constant controls in breeding farms to guarantee food safety for consumers.

❖ SECTION II

CHAPTER 1 – Whey

Whey, the liquid residue of cheese, casein and yoghurt production, is one of the biggest reservoirs of food protein available today. Of the total world milk output, the 11.4% is used for manufacturing cheese and approximately the 85% is discarded as whey. Whey is considered a waste product with very important nutritional properties, because it contains 55% milk nutrients. The annual production of whey is more than 120 million tonnes, with an estimated growth rate of 1–2% yearly. The majority of world whey production is not treated, the 20% is employed for the livestock feeding and only a small percentage, the 6.5% is utilized in the production of 'ricotta' (ISTAT/CLAL 2016). The amount of worldwide whey obtained from cheeses in the last years is shown in Table 11.

Table 11. Amount of worldwide whey obtained from cheeses (tons).

WORLD: AMOUNT OF WHEY OBTAINED FROM CHEESES (TONS)				
Year	**Milk production**	**Milk equivalent**	**Whey obtained from cheese**	**Powder whey obtained from cheese**
2018 (forecast)	17.595	151.910	129.124	8.135
2017	17.444	150.607	128.016	8.065
2016	17.533	151.375	128.669	8.106
2015	17.351	149.160	126.786	7.988
2014	16.889	145.157	123.383	7.773
2013	7.094	61.050	51.892	3.269
2012	7.036	60.266	51.226	3.227
Sorce: CLAL PROCESSING ON FAS-USDA DATA				

Whey can be classified into two categories as sweet whey and acidic whey. Sweet whey is produced during the making of rennet type of cheese like cheddar and Swiss cheese whereas acidic whey is a co-product from the process for the manufacture of certain acidic dairy products such as, cottage cheese and strained yoghurt. As reported by Tsakali *et al.* 2010, whey composition is widely dependent on the source of milk (cow, sheep, goat), the feed of the milk-producing animal, the processing method used and the stage of lactation.

1.1.Functional role of caseins and whey proteins

Milk proteins fraction can be divided into soluble and insoluble proteins. The soluble fraction of milk proteins is represented by whey proteins and the insoluble fraction by caseins. Traditionally, milk proteins consist of 80% casein and 20% whey proteins.

Casein is a phosphoprotein classified in five kinds of casein micelles that are different in molecular composition but are similar in structure (alpha S1 (αs1-CN), alpha S2 (αs2-CN), beta (β-CN), kappa (κ-CN) and (γ- CN) casein). Typically, they have a molecular weight between 14 and 25 kDa (Thorn, 2015). The principal role attributed to the insoluble fraction is the transport of calcium and phosphate due to their capacity to act as carriers. Furthermore, they are also responsible to support efficient stomach digestion of calcium and phosphorus. Additionally, caseins give origin to several bioactive peptides that have shown benefits in human health (Atamer *et al.* 2017). These include antioxidant, cytomodulatory, immunomodulatory, antihypertensive, and antithrombotic actions in the cardiovascular, nervous, immune, and digestive systems (Bhat *et al.* 2015).

Whey proteins are globular proteins having excellent nutritional properties which can be isolated from whey. The soluble fraction proteins include:

- β-lactoglobulin (β-Lg) is the main protein found in whey which represents 50% of whey proteins and also 12% of total proteins in milk. β-Lg has a molecular weight of 18 kDa and it is well-known protein for their capability to bind to fatty acids and retinol (vitamin A) and because of this, it has great foaming and gelation properties (Boland *et al.* 2014).
- α-lactalbumin (α–La) is the second most important protein content in whey and milk with a molecular weight of about 14 kDa. It comprises 20 % of total whey proteins and also 3.5 % of total proteins in milk. α–La has a high tryptophan (Trp) content which is useful for human brain function. α–La is known as a metalloprotein correlated on calcium (Ca^{2+}) ions (Derek *et al.* 2006).
- Lactoferrin (LF) is an 80 kDa member of the transferrin family of non-heme iron-binding monomeric glycoproteins (Arnold *et al.* 1980). LF is normally found in various liquids including milk, nasal, saliva. Lactoferrin is a crucial element in iron absorption and in exerting antioxidant, antimicrobial and anticarcinogenic effects (Giansanti *et al.* 2016, Orso *et al.* 2004, Tsuda *et al.* 2000).
- Bovine serum albumin (BSA) is another cow milk proteins with a molecular weight of 66 kDa. BSA has a significant biological effect on human health but it role in food and milk are not well known. BSA has only a slight effect on whey physiochemical properties due to its low concentration in milk. It is sometimes used as a protein standard in some experiments (Aguero *et al.* 2017).

- Immunoglobulin (Ig) has a molecular weight of 150-1000 kDa and represents the immunological part of the milk which can protect people against a wide range of bacteria and viruses. The highest amount of Ig is found in human milk whereas the low level is present in cow's milk. The immunoglobulins, most commonly found in the colostrum are essential to guarantee immunity to infants after birth (Hanson *et al.* 2003, Tovar Jiménez *et al.* 2012).

The amino acid profile is really different between the two milk fractions, in fact, whey is especially rich in branched chain amino acids, such as, leucine, isoleucine, and valine as well as lysine, whereas casein has a higher percentage of histidine, methionine, and phenylalanine (Wolfe *et al.* 2017). In addition to high-quality and biological effect, milk protein and bioactive compounds have shown the possible protective action in human health, include antibacterial, antiviral, antifungal, antioxidant, antihypertensive, antimicrobial, antithrombotic, opioid, and immunomodulatory effects (Bhat *et al.* 2015, Nongonierma *et al.* 2015, Park *et al.* 2015).

1.2. Possibility to whey reuse

Nowadays huge quantities of whey are one of the considerable disposal problems in the dairy industry which creates several environmental concerns, especially in developing countries. An investigation carried out in 11 cheese industry in Serbia has shown that 78.8 % of whey is discharged into river systems, contributing to the organic pollution of the environment. In recent years, environmental and economic problems associated with food waste have induced industries to reuse these materials in other preparations in order to minimize waste (Ostojic *et al.* 2005).

Over the past few decades, there has been an increased interest in the utilization of whey for the production of value-added products like whey

proteins. Several methods have been proposed for whey valorization, an interesting way to upgrade this effluent could be subjecting the whey to fermentation process because the biological wastewater treatment technologies is an expensive alternative (Arvanitoyannis & Giakoundis, 2006). Lactic acid bacteria (LAB) have the property of producing proteolytic enzymes that hydrolyze large milk protein producing small peptides and free amino acids, with several functional properties for human health (Wang *et al.* 2015).

1.3. Lactic acid bacteria in fermentation process

LABs are a heterogeneous group of microorganisms belonging to the *Lactobacillales*, *Propionibacterium* and *Bifidobacterium* strains. According to their hexose metabolic pathways, LABs are classified into two groups: homofermentative and heterofermentative. In the first case, lactic acid is the principal products and in the second case, others end products include acetic acid, carbon dioxide, or aroma compounds are produced in addition to lactic acid (Stanton *et al.* 2005). The production of lactic acid preserves foods by inhibition of the proliferation of undesirable microorganisms. In addition, lactic acid contributes to the formation of flavour and in the coagulation of caseins, contributing to modify the texture of fermented milk products (Papagianni *et al.* 2012).

LABs have been employed as a long time for fermentation of foods and underlie one of the oldest methods employed for food preservation. These microorganisms are suitable for dairy fermentation process because they have a proteolytic system, which decomposes casein and lactose digestive enzymes to obtain various products with numerous biological and antimicrobial activity. Furthermore, they have been known to produce

antagonistic compounds which inhibit food spoilage moulds and bacteria. Given the significant quantity of lactose contained in this by-product, it can be used directly as a substrate for the growth of different microorganisms. During fermentation, LAB releases a range of secondary metabolites, some of which have been associated with beneficial health effects (Panesar *et al.* 2012). The US Food and Drug Administration Agency has defined LAS as "generally recognized as safe GRAS status" (Burdock *et al.* 2004, FDA 2018).

1.4. Biological activity of compounds of fermented whey

Different bioactivities have been associated with whey proteins. Despite biological activities are present in the intact proteins, in various cases whey proteins act as precursors of bioactive peptides released during the hydrolysis of proteins through fermentation or enzymatic processes. (Yalcin, 2006; Hernández-Ledesma *et al.* 2008; Madureira *et al.* 2010). Among the bioactivities known so far, angiotensin-converting enzyme (ACE) inhibitory properties have received special attention due to their potential beneficial effects in the treatment of hypertension. Several published reports on ACE-inhibitory are associated with peptides derived from bovine milk (Hernández-Ledesma *et al.* 2002).

Gobbetti *et al.* 2000 have studied two different types of milk fermented by *L. delbrueckii subsp. bulgaricus* SS1 and *L. lactis subsp. Cremoris* FT4 which produced angiotensin-I-converting-enzyme inhibitory peptides. The antihypertensive activity of the milk fermented by *L. delbrueckii subsp. bulgaricus* SS1 was associated with sequences of β-casein (β-CN) fragments: f6–14, f7–14, f73–82, f74–82, and f75–82. Those from the milk fermented by L. lactis subsp. cremoris FT4 contained the sequences of β-CN: f7–14, f47–52, and f169–175 and k-CN: f155–160 and f152–160. Most of these sequences

showed analogies with antihypertensive sequences present in the literature. In particular, the β-CN f47–52 sequence had high homology than angiotensin-II (Nguyen *et al.* 2015).

As regard antioxidant activity, Xiao *et al.* 2015 have studied the antioxidant activity of soy whey after fermentation by *L. plantarum* in order to find out their nutraceutical potential. The analysis showed that the fermentation of whey has an effect on the total phenolic content (TPC), antioxidant activity and DNA damage protection. The results showed an increase in these activities compared with unfermented whey. In a study conducted by Qian *et al.* 2011 was evaluated the antioxidant, antihypertensive and immunomodulatory activity derived from milk fermented by two strains of bacteria: *L. delbrueckii ssp. bulgaricus LB34*. Bioactive compounds in the range from 5 to 3 kDa showed an appreciable antioxidant activity. The data obtained exhibited the potential of the milk fermented by *L. delbrueckii ssp. bulgaricus LB340* as a functional food.

The antimicrobial effect obtained from the bioactive compounds of whey may be releated to the different active molecules relased during fermentation process as reported by scientific studies. Acids are in general responsible for the antimicrobial effect due to their interfering nature with the maintenance of the cell membrane potential, the capability to inhibit a variety of metabolic functions or supply an hostile acid environment for the growth of pathogenic and deteriorating microorganisms (Reis *et al.* 2012). In addition, the production of phenolic acids could have a secondary synergistic effect on the inhibition of fungal growth of whey fermented by LABs. Peyer *et al.* 2016 studied the effect of carboxylic acids, composed of both organic and phenolic acids, released in a barley malt substrate fermented by LAB highlighting the inhibitory effect of these compounds on fungal growth.

Hati *et al.* 2018 have studied growth, proteolysis and antimicrobial activity of LAB in a skimmed milk medium supplemented with different concentrations of whey protein. Finally, the antimicrobial peptide in the fermented milk was identified as lactoferrin by RP-LC/MS analysis. Several scientific studies have shown that lactoferrin exerts several beneficial health effects on humans and animals such as antimicrobial, antiinfective, anti-cancer and anti-inflammatory effects (El-Loly *et al.* 2011).

Gerez *et al.* 2013 have evaluated the potential of ninety-one LAB strains; ten out of the total of tested strains were selected due to their high inhibitory effect in fungal growth. In the cell-free supernatants, lactic, acetic and phenyllactic acids were identified as metabolites responsible for an antifungal effect. The antifungal activity was lost after the neutralization treatment determining the acidic nature of the antifungal metabolites. Inactivation of fungal growth by lactic, acetic and other organic acids is a well-known fact. This piece of evidence has also been reported by other researchers (Stanojević-Nikolić*et al.* 2016).

The traditional view of whey as a by-product of dairy industry with little value has disappeared, and now the whey is seen as a potential source of bioactive components that can be used in the formulation of multiple functional foods.

1.5. Application of bioactive compounds of whey to increase the shelf-life of bread

Nowadays, research efforts are generally aimed at improving quality with respect to a longer shelf-life. There is a significant need to improve food preservation method. Several bacterial species that contaminate foodstuffs and crops are pathogenic and may cause a range of food-borne illnesses

(Behravesh *et al.* 2012). Fungal spoilage can also lead to serious economic losses. It has been estimated that about one quarter of the world's food supply is lost as a result of microbial spoilage, and food-borne microbial infections represent a constant and serious threat to human health. Indeed, the most common type of microbial spoilage is mould growth that contributes to the shortening of products' shelf life (Garnier *et al.* 2017).

The traditional method of preserving food from the effect of microbial growth include thermal processing, freezing, refrigeration, drying, irradiation and addition of antimicrobial agents or salts in food. Antimicrobials are used to enhance the quality and safety of food by reducing surface contamination of processed food. Antimicrobials reduce the population of microorganism by extending the lag phase of the microorganism or through their inactivation (Sharif *et al.* 2017, Hintx *et al.* 2015, Charozo *et al.* 2015).

Among different food products, bakery products comprise an important part of a balanced diet and for this reason, a wide variety of such products can be found on food market shelves. Fungi are the most common spoilage organisms in baked products such as bread; the shelf life without preservatives is 3–4 days. Like many processed foods, bakery products are subjected to physical, chemical and microbial spoilage. Physical and chemical spoilage limits the shelf-life of low and intermediate moisture bakery products, whereas microbial spoilage is the concern in high moisture products (aw > 0.85) (Smith *et al.* 2004).

In wheat based bakery products, deteriorations have been attributed to the following fungi genus: *Penicillium*, *Aspergillus*, *Cladosporium* and *Neurospora*. In wheat bread, *P. commune, P. solitum, P. Corylophilum,* and *A. flavus* are the

most dominant. *P. Roqueforti, P. Corylophilum*, and *Eurotium* species are more dominant in rye bread. Microorganisms may be present in raw materials, developed in the products or contaminate it during processing and packaging (Garcia *et al.* 2018).

Microbiological shelf-life of bread is generally prolonged by the use of the chemical preservative like calcium propionate (Belz *et al.* 2012). In Europe, the maximum limits for propionic acid and propionate have been established at 3000 mg/kg in pre-packed sliced bread and rye bread, 2000 mg/kg in energy reduced bread, partially/pre-baked bread, pre-packed rolls and buns and 1000 mg/kg in pre-packed bread (EFSA, 2014).

However, as a result of increasing signs of negative effects on consumer health, biopreservation strategies are given further attention (Dengate & Ruben, 2002). The rationale of incorporating antimicrobials into bread preparation is to prevent surface growth in foods where a large portion of spoilage and contamination occurs. There is a clear trend toward preventing or delaying microbial growth in foodstuffs by means of natural preservatives. Due to its unique properties, whey can be an excellent medium for bacteria and enzymes in the laboratory (Karwowska *et al.* 2014; Ribes *et al.* 2018, Jideani *et al.* 2016).

1.6. Identification of phenolic compounds

For the identification of the phenolic compounds, I used a mass spectrometer with a Q-TOF detector. TOF is a relatively mature technology proposed at the first time by Stephens in 1946 and subsequently developed by Cameron and Eggers two years later (Cameron & Eggers, 1948). TOF instrument allows of ions separation based on mass-to-charge (m/z) ratio which travels through a flight tube: the lighter ions on will fly faster and reach the detector

earlier. At the moment, there are two principal types of TOF analyzers: linear and of orthogonal acceleration, even if linear time-TOFs is the most commonly used configuration nowadays. The TOF technique has been employed for various years in different areas, particularly for quantification of large molecules. The analysis in TOF-MS mode gives the greatest flexibility and throughput due to its speed of acquisition, high resolution, and its ability to acquire large scan windows (>20000 amu). On the other hand, the use of TOF-MS is limited by the sensitivity. The main weaknesses of TOF detectors are correlated with saturation from a threshold intensity which affects the measured signal amplitude and also can result in a relevant mass shift of the ions being affected by saturation. Even if this mode produces the highest signal, and no signal loss due to the poor fragmentation efficiency of most compounds, chromatograms are usually affected with high chemical noise, which reduces the signal-to-noise ratio (S/N)(Ramagiri *et al.* 2012, García-Reyes *et al.* 2017).

References:

- Aguero, R., Bringas, E., San Román, M. F., Ortiz, I., & Ibanez, R. (2017). Membrane Processes for Whey Proteins Separation and Purification. A Review. *Current Organic Chemistry*, 21(17), 1740-1752.
- Arnold, R. R., Brewer, M., & Gauthier, J. J. (1980). Bactericidal activity of human lactoferrin: sensitivity of a variety of microorganisms. *Infection and immunity*, 28(3), 893-898.
- Arvanitoyannis, I. S., & Giakoundis, A. (2006). Current strategies for dairy waste management: a review. *Critical reviews in food science and nutrition*, 46(5), 379-390.
- Atamer, Z., Post, A. E., Schubert, T., Holder, A., Boom, R. M., & Hinrichs, J. (2017). Bovine β-casein: Isolation, properties and functionality. A review. *International Dairy Journal*, 66, 115-125.
- Behravesh, C. B., Williams, I. T., & Tauxe, R. V. (2012). Emerging foodborne pathogens and problems: expanding prevention efforts before slaughter or harvest
- Belz, M. C., Mairinger, R., Zannini, E., Ryan, L. A., Cashman, K. D., & Arendt, E. K. (2012). The effect of sourdough and calcium propionate on the microbial shelf-life of salt reduced bread. *Applied microbiology and biotechnology*, 96(2), 493-501.
- Bhat, Z. F., Kumar, S., & Bhat, H. F. (2015). Bioactive peptides of animal origin: a review. *Journal of food science and technology*, 52(9), 5377-5392.
- Boland, M., Singh, H., & Thompson, A. (Eds.). (2014). *Milk proteins: From expression* to food. Academic Press.
- Burdock, G. A., & Carabin, I. G. (2004). Generally recognized as safe (GRAS): history and description. *Toxicology letters*, 150(1), 3-18.
- Cameron, A. E., & Eggers, D. F., Jr. (1948). An ion "velocitron". *Review of Scientific Instruments*, 19, 605e647
- Carocho, M., Morales, P., & Ferreira, I. C. (2015). Natural food additives: Quo vadis?. *Trends in Food Science & Technology*, 45(2), 284-295.
- CLAL 2016 Processing on data FAS-USD https://www.clal.it/mini_index.php?section=produzione_cheese_siero&mode=mini&h=450&w=950.
- Dengate, S., & Ruben, A. (2002). Controlled trial of cumulative behavioural effects of a common bread preservative. *Journal of Paediatrics and Child Health*, 38(4), 373-376.

- Dereck E.W. Chattertona , Geoffrey Smithersb , Peter Roupasb , Andre´ Brodkorbc (2006). Bioactivity of b-lactoglobulin and a-lactalbumin—Technological implications for processing. *International Dairy Journal*. 16 1229–1240
- EFSA Panel on Food additives and Nutrient Sources added to Food (ANS). (2014). Scientific Opinion on the re-evaluation of propionic acid (E 280), sodium propionate (E 281), calcium propionate (E 282) and potassium propionate (E 283) as food additives. *EFSA Journal*, 12(7), 3779.
- El-Loly, M. M., & Mahfouz, M. B. (2011). Lactoferrin in relation to biological functions and applications: a review. *International Journal of Dairy Science* 6(2), 79-111.
- European Food Safety Authority (EFSA). (2009). Review of the potential health impact of β-casomorphins and related peptides. *EFSA Journal*, 7(2), 231r.
- FDA, G. substances (SCOGS) database (No. 46a). Report.https://www.fda.gov/food/ingredientspackaginglabeling/gras/scogs/default.htm
- Garcia, M. V., da Pia, A. K. R., Freire, L., Copetti, M. V., &Sant'Ana, A. S. (2018). Effect of temperature on inactivation kinetics of three strains of *Penicillium* paneum and *P. roqueforti* during bread baking. *Food Control.*
- García-Reyes, J. F., Moreno-González, D., Nortes-Méndez, R., Gilbert-López, B., & Díaz, A. M. (2017). HRMS: Hardware and Software. *In Applications in High Resolution Mass Spectrometry* (pp. 15-57).
- Garnier, L., Valence, F., & Mounier, J. (2017). Diversity and Control of Spoilage Fungi in Dairy Products: An Update. *Microorganisms*, 5(3), 42.
- Gerez, C. L., Torres, M. J., De Valdez, G. F., & Rollán, G. (2013). Control of spoilage fungi by lactic acid bacteria. *Biological Control*, 64(3), 231-237.
- Giansanti, F., Panella, G., Leboffe, L., & Antonini, G. (2016). Lactoferrin from milk: nutraceutical and pharmacological properties. *Pharmaceuticals*, 9(4), 61.
- Gobbetti, M., Ferranti, P., Smacchi, E., Goffredi, F., & Addeo, F. (2000). Production of Angiotensin-I-converting-enzyme-inhibitory peptides in fermented milks started by Lactobacillus delbrueckiisubsp. bulgaricus SS1 and Lactococcus lactissubsp. cremoris FT4. *Applied and Environmental Microbiology*, 66(9), 3898-3904.
- Hanson, L. Å., Korotkova, M., Lundin, S., Håversen, L., SILFVERDAL, S. A., MATTSBY-BALTZER, I. N. G. E. R. & Telemo, E. (2003). The transfer of immunity from mother to child. *Annals of the New York Academy of Sciences*, 987(1), 199-206.

- Hati, S., Patel, N., Sakure, A., & Mandal, S. (2018). Influence of whey protein concentrate on the production of antibacterial peptides derived from fermented milk by lactic acid bacteria. *International Journal of Peptide Research and Therapeutics*, 24(1), 87-98.
- Hernández-Ledesma, B., Recio, I., & Amigo, L. (2008). β-Lactoglobulin as source of bioactive peptides. *Amino acids*, *35*(2), 257-265.
- Hernández-Ledesma, B., Recio, I., Ramos, M., & Amigo, L. (2002). Preparation of ovine and caprine β-lactoglobulin hydrolysates with ACE-inhibitory activity. Identification of active peptides from caprine β-lactoglobulin hydrolysed with thermolysin. *International Dairy Journal*, 12(10), 805-812.
- Hintz, T., Matthews, K. K., & Di, R. (2015). The use of plant antimicrobial compounds for food preservation. *BioMed research international*, 2015.
- Jideani, V. A., & Vogt, K. (2016). Antimicrobial packaging for extending the shelf life of bread—a review. *Critical reviews in food science and nutrition*, 56(8), 1313-1324.
- Karwowska, E., Andrzejewska-Morzuch, D., Łebkowska, M., Tabernacka, A., Wojtkowska, M., Telepko, A., & Konarzewska, A. (2014). Bioleaching of metals from printed circuit boards supported with surfactant-producing bacteria. *Journal of hazardous materials*, 264, 203-210.
- Madureira, A. R., Tavares, T., Gomes, A. M. P., Pintado, M. E., & Malcata, F. X. (2010). Invited review: physiological properties of bioactive peptides obtained from whey proteins. *Journal of dairy science*, *93*(2), 437-455.
- Nguyen, D. D., Johnson, S. K., Busetti, F., & Solah, V. A. (2015). Formation and degradation of beta-casomorphins in dairy processing. *Critical reviews in food science and nutrition*, 55(14), 1955-1967.
- Nongonierma, A. B., & FitzGerald, R. J. (2015). Bioactive properties of milk proteins in humans: A review. *Peptides*, 73, 20-34.
- Orsi, N. (2004). The antimicrobial activity of lactoferrin: current status and perspectives. *Biometals*, 17(3), 189-196.
- Ostojić, S., Pavlović, M., Živić, M., Filipović, Z., Gorjanović, S., Hranisavljević, S., & Dojčinović, M. (2005). Processing of whey from dairy industry waste. *Environmental Chemistry Letters*, 3(1), 29-32.
- Panesar, P. S., & Kennedy, J. F. (2012). Biotechnological approaches for the value addition of whey. *Critical reviews in biotechnology*, 32(4), 327-348.

- Papagianni, M. (2012). Recent advances in engineering the central carbon metabolism of industrially important bacteria. *Microbial cell factories*, 11(1), 50.
- Park, Y. W., & Nam, M. S. (2015). Bioactive peptides in milk and dairy products: a review. *Korean journal for food science of animal resources*, 35(6), 831.
- Peyer, L. C., Axel, C., Lynch, K. M., Zannini, E., Jacob, F., & Arendt, E. K. (2016). Inhibition of *Fusarium* culmorum by carboxylic acids released from lactic acid bacteria in a barley malt substrate. *Food Control*, 69, 227-236.
- Qian, B., Xing, M., Cui, L., Deng, Y., Xu, Y., Huang, M., & Zhang, S. (2011). Antioxidant, antihypertensive, and immunomodulatory activities of peptide fractions from fermented skim milk with Lactobacillus delbrueckii ssp. bulgaricus LB340. *Journal of Dairy Research*, 78(1), 72-79.
- Ramagiri, S., & Garofolo, F. (2012). Large molecule bioanalysis using Q-TOF without predigestion and its data processing challenges. *Bioanalysis*, 4(5), 529-540.
- Reis, J. A., Paula, A. T., Casarotti, S. N., & Penna, A. L. B. (2012). Lactic acid bacteria antimicrobial compounds: characteristics and applications. *Food Engineering Reviews*, 4(2), 124-140.
- Ribes, S., Fuentes, A., Talens, P., & Barat, J. M. (2018). Prevention of fungal spoilage in food products using natural compounds: a review. *Critical reviews in food science and nutrition*, 58(12), 2002-2016.
- Sharif, Z. I. M., Mustapha, F. A., Jai, J., & Zaki, N. A. M. (2017). Review on methods for preservation and natural preservatives for extending the food longevity. *Chemical Engineering Research Bulletin*, 19.
- Smith, J. P., Daifas, D. P., El-Khoury, W., Koukoutsis, J., & El-Khoury, A. (2004). Shelf life and safety concerns of bakery products—a review. *Critical reviews in food science and nutrition*, 44(1), 19-55.
- Stanojević-Nikolić, S., Dimić, G., Mojović, L., Pejin, J., Djukić-Vuković, A., & Kocić-Tanackov, S. (2016). Antimicrobial activity of lactic acid against pathogen and spoilage microorganisms. *Journal of food processing and preservation*, 40(5), 990-998.
- Stanton, C., Ross, R. P., Fitzgerald, G. F., & Van Sinderen, D. (2005). Fermented functional foods based on probiotics and their biogenic metabolites. *Current opinion in biotechnology*, 16(2), 198-203.
- Stephens, W. E. (1946). Proceedings of the American Physical Society. *Physical Reviews*, 69, 691.

- Thorn, D. C., Ecroyd, H., Carver, J. A., & Holt, C. (2015). Casein structures in the context of unfolded proteins. *International Dairy Journal*, 46, 2-11.
- Tovar Jiménez, X., Arana Cuenca, A., Téllez Jurado, A., Abreu Corona, A., & Muro Urista, C. R. (2012). Traditional methods for whey protein isolation and concentration: effects on nutritional properties and biological activity. *Journal of the Mexican Chemical Society*, 56(4), 369-377.
- Tsakali, E., Petrotos, K., D'Allessandro, A., & Goulas, P. (2010, June). A review on whey composition and the methods used for its utilization for food and pharmaceutical products. In *6th International conference on simulation and modelling in the food and bio-industry FOODSIM*.
- Tsuda, H., Sekine, K., Ushida, Y., Kuhara, T., Takasuka, N., Iigo, M., & Moore, M. A. (2000). Milk and dairy products in cancer prevention: focus on bovine lactoferrin. Mutation Research/Reviews in Mutation Research, 462(2), 227-233.
- Wang, Y., Tashiro, Y., & Sonomoto, K. (2015). Fermentative production of lactic acid from renewable materials: recent achievements, prospects, and limits. *Journal of bioscience and bioengineering*, 119(1), 10-18.
- Wolfe, R. R. (2017). Branched-chain amino acids and muscle protein synthesis in humans: myth or reality?. *Journal of the International Society of Sports Nutrition*, 14(1), 30.
- Xiao, Y., Wang, L., Rui, X., Li, W., Chen, X., Jiang, M., & Dong, M. (2015). Enhancement of the antioxidant capacity of soy whey by fermentation with *Lactobacillus plantarum* B1–6. *Journal of Functional Foods*, 12, 33-44.
- Yalcin, A. S. (2006). Emerging therapeutic potential of whey proteins and peptides. *Current pharmaceutical design*, *12*(13), 1637-1643.

CHAPTER 2 - Objectives section II

2.1. General Objectives section II

The present study aims to evaluate the antifungal activity of fermented whey containing lactic acid bacteria and to apply the latter to the preparation of pita bread, in order to extend the storage time of the product. I have also evaluated the biological activities of this by-product for use it in a formulation of a potential nutraceutical as shown in Figure 3 (work in progress).

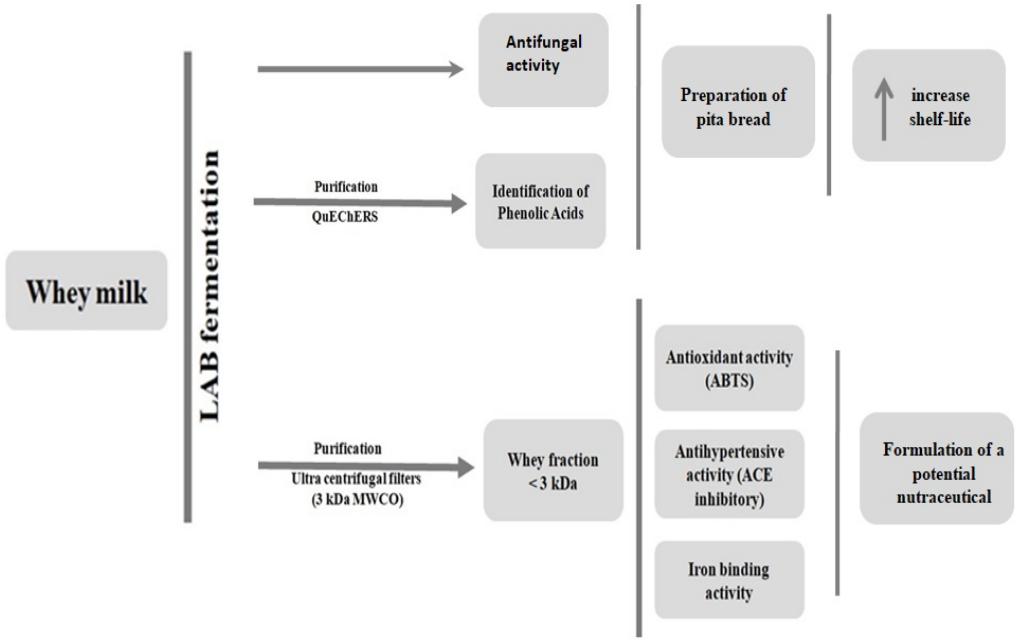


Figure 3. General objective of the second part of my thesis work: whey treatments; fermentation at three different times (24, 48, 72h), purification on centricon amicon with cut-off <3kDa and evaluation of biological and antimicrobial proprieties of this particular fraction.

2.2. Specific Objectives section II

1. Sample preparation: determination of macronutrient composition of by-product (protein, fat, lactose, humidity and ash), pasteurization and fermentation;

2. Qualitative evaluation of the antifungal activity of cell-free supernatant (CFS) obtained from fermented whey with lactic acid bacteria and quantitative determination of the antifungal activity of CFS by defining the MIC and the MFC;

3. Evaluation of inhibition of fungal growth in bread, by inserting fermented whey with lactic acid bacteria in its preparation.

4. Evaluation of biological activities including ACE-inhibitory, antioxidants and Fe-binding in order to use this by-product for the formulation of a nutraceutical.

SPECIFIC OBJECTIVE 1

Sample preparation: determination of macronutrient composition of by-product (protein, fat, lactose, humidity and ash), pasteurization and fermentation.

Materials and methods

Chemicals and reagents

Potassium iodide solution, sodium thiosulphate, bradford reagent, chloramine T, tungstate acid, phenylmethanesulfonyl fluoride, starch and glycerol were purchased from Sigma Aldrich (Milan, Italy). Hydrochloric acid was acquired from Merck (Darmstadt, Germany). All other chemicals and reagents were of analytical grade. Deionized water (<18 MX cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Standard of bovine serum albumin (BSA), was acquired from Sigma Aldrich (Milan, Italy). Buffered peptone water, potato dextrose agar (PDA), potato dextrose broth (PDB), De Man Rogosa-Sharpe medium (MRS Broth), were provided by Oxoid (Madrid, Spain).

Microorganisms and culture conditions

L. plantarum (CECT 220; 221; 223; 748) were obtained by Colección Espanola de Cultivos Tipo (CECT) and stored at −80°C in glycerol 25%. The cultures were defrosted under sterile conditions, diluted in sterile MRS broth and finally incubated at 37 °C during 48 h.

Freeze-dried whey was provided by the Nutriops company, S.L. (Murcia, Spain). This powder whey was obtained from cow's milk without chemical preservatives. Liquid sweet whey was provided by the ALCLIPOR company, S. A. L. (Benassal, Spain). This whey was obtained from goats's milk without

chemical preservatives. Sample were stored at 4 °C until analysis carried out within 48 h after arrival of sample.

Pasteurization of whey

The standard method was simulated by heating sweet whey in a water bath to 63 °C and maintaining that temperature for 30 min with continuous mixing, followed by rapid cooling according to standardized guidelines (Roth-Walter*et al* .2008).

Whey fermentation

Freeze-dried whey was fermented with three different species of lactobacilli (*L. plantarum* CECT 220; 221; 748) for 24, 48 and 72 hours at 37 °Cusing the method of Luz&Izzo *et al.* 2018 with some modifications. Whey was reconstituted with distilled water to a 10% (w/v) final concentration. After pasteurization at 65 °C for30 minutes, 30 mL were fermented in sterile conditions with1 mL of previously activated bacteria of *L. plantarum* (CECT 220; 221; 748). Liquid sweet whey was fermented with four different strains of lactobacilli (*L. plantarum* CECT 220; 221; 223; 748) only for 72 h at 37 °C. The suspension of each LAB at concentration of 10^8 CFU/mL were added to 40 mL of sterile pasteurized sweet whey.

After then, fermented whey was centrifuged at 4000 rpm for 10 min to remove bacteria (Eppendorf 5810R, Hamburg, Germany). The cell-free-supernatant (CFS) was collected in the tube and lyophilized after adding to protease inhibitor 1mM PMSF (phenylmethanesulfonyl fluoride). The CFS were lyophilized and stored at −80 °C for further analysis. A schematic representation of fermentation process for obtaining the cell-free supernatant is shown in figure 4.

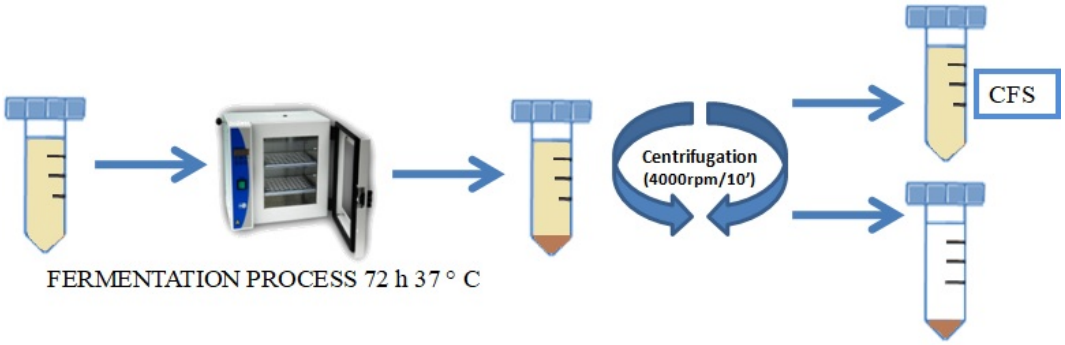


Figure 4. Schematic representation of fermentation process for obtaining the cell-free supernatant.

Characterization of raw material

The determination of macronutrient composition (protein, fat, lactose, humidity and ash) was performed according to the guidelines instructions listed below.

Soxhlet extraction

Freeze-dried sweet whey (5.00 g) was extracted with n-hexane in a Soxhlet apparatus by a continuous series of cycles of boiling and condensation of solvent for 6 h (Teixeira *et al.* 2018).

Protein determination

Protein determination was performed using the method of Bradford with a slight modification to the original assay (Zor *et al.* 1996). A volume of 0.1 mL of dye reagent was added to 0.1 mL of appropriately diluted sample. After mixing and incubated in the dark, the absorbance readings at 595 nm were taken between 5 and 60 min. The mean of replicate solvent blanks was subtracted from the mean of replicate samples. The BSA (3.12-15 µg/mL) was used as standard to quantifie the proteins in the sample.

Lactose determination

Lactose determination was carried out using the chloramine T assay. A ten mL aliquot of sweet whey was added 25 mL of water and 40 mL of deproteinization reagent (tungstate acid) and making up to 100 mL with water. The mixture was vortexed vigorously and filtrated after 1 h of the pose in the dark conditions. Briefly, ten mL of sample were introduced into a flask of 250 mL and five mL of 10 % (w/v) potassium iodide solution and 20 mL of chloramine T 0.040M were added. The mixture was shaken for 90 min in dark conditions. Then, five mL of 2M hydrochloric acid were added to the flask and titrated with 0.04M sodium thiosulphate to a starch (1%) end point. A blank titration was performed under same conditions. The amount of the lactose present in the measured aliquot was accurately calculated from the volume of chloramine that has reacted with the sample (Silvestre *et al.* 2014).

Humidity and ash

Humidity were measured after drying 40 mL aliquots of thoroughly mixed samples at 100±2 °C for 3 h in porcelain crucibles until reaching the constant weight. Ash was further incinerated at 450 °C for 12 h for determination of ash content. Results were expressed in mg/100 mL (Ranganna *et al.* 1986).

Density and pH measurement

Density value was assessed using a lactodensimeter (Lactometer Quevenne, Brannan Thermometers). pH value was determined using a basic 20 pHmeter (Crison, Spain).

Results and discussion

Pasteurization and fermentation processes

The process of milk pasteurization is intended to ensure the safety of the product reducing the number of viable spoilage microorganisms, and inactivating several milk enzymes, as consequence of this process an increase in the shelf-life was obtained. Shelf life is influenced by multiple factors such as the quality of raw milk, post-pasteurization contamination especially during packaging, light exposure, and temperature control during storage and distribution. Post-pasteurization contamination is caused by improperly cleaned and sanitized cooling sections, process lines, valves, tanks, and packaging materials (Schmidt *et al.* 2008).

Pasteurization is a mild type of heat treatment which causes only minor changes in milk flavor and nutritional quality. Raw milk spoils within a few days even when stored at refrigeration temperature, whereas pasteurized milk can have a shelf life between 7 and 28 days, or even longer. The goal of pasteurization is to achieve 99.999% reduction in viable microorganisms. Pasteurization should not be confused with sterilization, where the objective is to kill all microorganisms (pathogenic and spoilage) in the food (Claeys *et al.* 2013).

However, in view of the above I performed pasteurization of goat's whey before the fermentation process using LAB. As reported by other authors, the bioconversion of lactose present in whey to valuable products has been actively explored. Since whey contains significant quantities of lactose, as also reported of the macronutrients analysis, an interesting way to upgrade this effluent could be as a substrate for fermentation (Panesar *et al.* 2007).

Evaluation of compositional analysis

The nutritional composition of two typologies of whey is shown in Table 12. The protein fractions of bovine and goat's milk are qualitatively very similar, and the major difference among these is related to the proportions and classes of caseins. In the protein profile of goat's whey low concentration or absence of α-s1 casein was reported. Studies involving milk from various animal species, including goats, indicate that milk and whey proteins, as well as the peptides generated from these proteins, have important biological activities, such as antimicrobial, immunomodulatory, antioxidant, antithrombotic, hypocholesterolemic and antihypertensive activities. Milk proteins and its derivates maybe an important source of antimicrobial peptides, natural agents having potential application as biopreservatives in food matrices (Hejtmánková *et al.* 2012; da Costa *et al.* 2014). As reported by da Costa, who analyzed protein composition of goat's milk in order to evaluate the antibacterial activity of its protein fractions, only the protein fraction containing the largest amounts of casein (60–90%) are able to inhibit bacterial growth, with MIC values between 50 and 100 mg/mL.

Table 12. Macronutrient composition of goat's liquid whey and cow's freeze dried whey utilized in fermentation process with *Lactobacillus plantarum.*

Macronutrient composition of whey	GOAT'S LIQUID SWEET WHEY	COW'S FREEZE DRIED WHEY
	g/100 g	g/100g
Lipids	0.9	0.9
Carbohydrates		
- Lactose	69.8	71.5
Proteins	14.7	12.2
Ash	0.7	2

References:

- Claeys, W. L., Cardoen, S., Daube, G., De Block, J., Dewettinck, K., Dierick, K., & Vandenplas, Y. (2013). Raw or heated cow milk consumption: Review of risks and benefits. *Food Control*, *31*(1), 251-262.
- da Costa, W. K. A., de Souza, E. L., Beltrao-Filho, E. M., Vasconcelos, G. K. V., Santi-Gadelha, T., de Almeida Gadelha, C. A., & Magnani, M. (2014). Comparative protein composition analysis of goat milk produced by the Alpine and Saanen breeds in Northeastern Brazil and related antibacterial activities. *PLoS One*, *9*(3), e93361.
- Hejtmánková, A., Pivec, V., Trnková, E., & Dragounová, H. (2012). Differences in the composition of total and whey proteins in goat and ewe milk and their changes throughout the lactation period. *Czech J. Anim. Sci*, *57*(7), 323-331.
- Izzo, L., Luz, C., Graziani, G., Gaspari, A., Ritieni, A., Mañes, J., & Meca, G. (2018). Evaluation of biological and antimicrobial properties of freeze-dried whey fermented by different strains of Lactobacillus plantarum. *Food & Function*.
- Panesar, P. S., Kennedy, J. F., Gandhi, D. N., & Bunko, K. (2007). Bioutilisation of whey for lactic acid production. *Food chemistry*, *105*(1), 1-14.
- Ranganna, S. (1986). Handbook of analysis and quality control for fruit and vegetable products. *Tata McGraw-Hill Education.*
- Roth-Walter, F., Berin, M. C., Arnaboldi, P., Escalante, C. R., Dahan, S., Rauch, J., & Mayer, L. (2008). Pasteurization of milk proteins promotes allergic sensitization by enhancing uptake through Peyer's patches. Allergy, 63(7), 882-890.
- Schmidt, R. H. (2008). Microbiological considerations related to dairy processing. *Dairy processing and quality assurance*, 105-143.
- Silvestre, D., Fraga, M., Gormaz, M., Torres, E., & Vento, M. (2014). Comparison of mid-infrared transmission spectroscopy with biochemical methods for the determination of macronutrients in human milk. *Maternal&childnutrition*, *10*(3), 373-382.
- Teixeira, G. L., Ghazani, S. M., Corazza, M. L., Marangoni, A. G., & Ribani, R. H. (2018). Assessment of subcritical propane, supercritical CO2 and Soxhlet extraction of oil from sapucaia (Lecythispisonis) nuts. *The Journal of Supercritical Fluids*, 133, 122-132.

- Zor, T., &Selinger, Z. (1996). Linearization of the Bradford protein assay increases its sensitivity: theoretical and experimental studies. *Analytical biochemistry*, 236(2), 302-308.

SPECIFIC OBJECTIVE 2

Qualitative evaluation of the antifungal activity of cell-free supernatant (CFS) obtained from fermented whey with lactic acid bacteria and quantitative determination of the antifungal activity of CFS by defining the MIC and the MFC of two different type of whey:

1. Freeze-dried cow's whey (FDW);
2. Liquid sweet goat's whey (SW).

Materials and methods

Chemicals and reagents

Sodium chloride, magnesium sulfate were purchased from Sigma Aldrich (Milan, Italy). Acetonitrile, methanol, formic acid, HPLC grade were acquired from Merck (Darmstadt, Germany). All other chemicals and reagents were of analytical grade. Syringe filters with nylon membrane (NY, 15 mm, diameter 0.22 μm) were supplied by Phenomenex (Castel Maggiore, Italy).

Standard of gallic, protocatechuic, chlorogenic, caffeic, syringic, vanillin, p-coumaric, ferulic, hydroxibenzoic, vanillic, sallicilic, hydrocinnamic, sinapic, benzoic, DL-3-phenyllactic, 1-2 dihydroxybenzene, 3,4-dihydroxyhydrocinnamic, DL-p-hydroxyphenyllatic acids (purity >98%) were acquired from Sigma Aldrich (Milan, Italy). Buffered peptone water, potato dextrose agar (PDA), potato dextrose broth (PDB), De Man Rogosa-Sharpe medium (MRS Broth), were provided by Oxoid (Madrid, Spain).

Deionized water (<18 MX cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Buffered peptone water,

potato dextrose agar (PDA), potato dextrose broth (PDB), De Man Rogosa-Sharpe medium (MRS Broth), were provided by Oxoid (Madrid, Spain).

Microorganisms and culture conditions

L. plantarum (CECT 220; 221; 223; 748) were obtained from Colección Espanola de Cultivos Tipo (CECT) and stored at −80 °C in glycerol 25%. The cultures were defrosted under sterile conditions, diluted in sterile MRS broth and finally incubated at 37 °C for 48 h.

For the evaluation of the antifungal activity, the mycotoxigenic strains used *P. expansum CECT 2278, P. brevicopactum CECT 2316, P. camemberti CECT 2267, P. roqueforti CECT 2905, P. verrucosum VTT D-01847, P. digitatum CECT 2954, P. nordicum CECT 2320, P. pinophilum CECT 2912, P. commune CECT 20767, P. solitum CECT 20818; A. parasiticus CECT 2681, A. flavus ITEM 8111, A. niger CECT 2088, A. steynii CECT 20510, A. carbonarius ITEM 5010, A. brasiliensis CECT 2574, A. sclerotioniger CECT 20583, A. tubingensis CECT 20543, A. tubingensis CECT 20544, A. lacticoffeatus CECT 20581; F. moniliforme CECT 2987, F. moniliforme CECT2152,* F. *moniliformis* CECT2982, *F. graminearum* CECT2049, *F. graminearum ITEM 6415, F. proliferatum ITEM 12072, F. verticillioides ITEM 12052, F. verticillioides ITEM 12043, F. verticillioides* CECT 20926, *F. verticillioides ITEM 12044, F. sporotrichioides ITEM 12168, F. langsethiae ITEM 11031, F. poae ITEM 9151* were purchased from the Colección Espanola de CultivosTipo (CECT). All strains were maintained in PDA at room temperature (25 °C) until use.

Antifungal activity test

Antifungal activity of fermented whey was assessed by using Kirby-Bauer test according to Varsha *et al.* 2014 with slight modifications. The analysis was performed against three different strains of fungi belonging to the

Penicillium, *Aspergillus* and *Fusarium* genera at 24, 48 and 72 h for freeze-dried whey and after 72 h of fermentation time for goat's whey. Briefly, 70 µL of sample (0.5 g/L) solubilized in potato dextrose broth (PDB) were added to the wells (5 mm diameter) cut on potato dextrose agar which was previously spread plated with 1×10^4 fungal spores and kept at 26 °C for 3 days (Figure 5). Following incubation, the zones of inhibition were measured. Overall, halos equal to or greater than 8-10 mm were considered positive for antifungal activity (Nascimento *et al.* 2000).

Determination of minimum inhibitory concentration and minimum fungicidal concentration (MIC–MFC)

The antifungal activity of fermented whey by *L. plantarum* strains at 72 h was determined using the method of Fothergill *et al.* 2012 with some modifications. The 96-well sterile microplates were utilized to perform the experiment. A negative control without adding of spores and a positive control in the presence of spores were included (Figure 5). The assay was performed by a micro-dilution technique to obtain serial dilution concentrations. First, 0.5 g/L of the sample was diluted in the same volume (100 µL) of spores at a concentration of 5×10^4 to obtain a final concentration range from 250 g/L to 1.95 g/L. The microplates were incubated at 26 °C for 72 h. The micro-assay were performed in triplicate. The lowest concentration of sample capable to inhibit the visible growth of microorganisms is known as MIC. The minimum fungicidal concentration (MFC) was determined by planting 10 µL of each different concentrations with a visible growth of the subculture of the sample in the PDA plate. The MFC value was determined after 72 h of incubation at 26 °C.

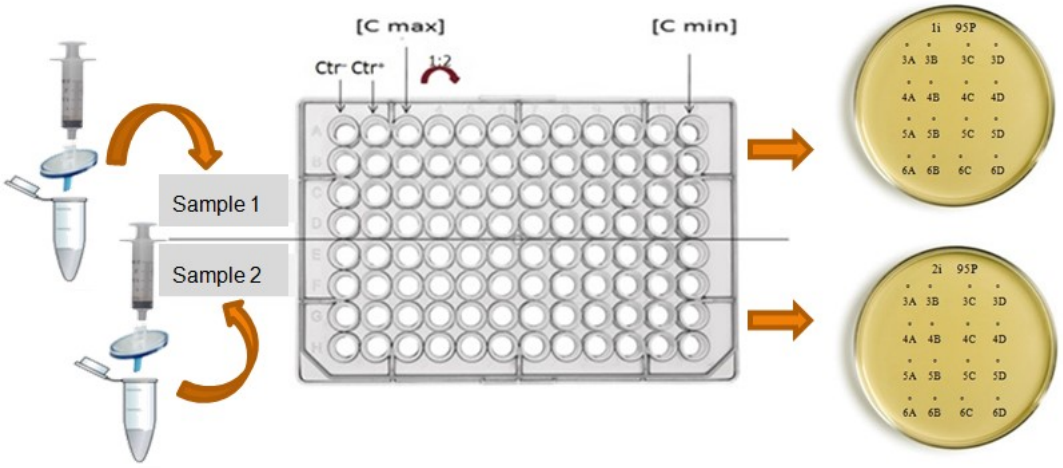


Figure 5. In the picture is shown a 96-well sterile microplate utilized to perform the experiment of antifungal activity.

LC-ESI-qTOF-MS separation and identification of phenolic acids

The extraction of the phenolic compounds was performed using the method described by Brosnan *et al.* 2014 with some modifications. Ten milliliters of fermented whey were extracted with 10 mL of 1% of formic acid in ethyl acetate, 4 g of $MgSO_4$ and 1 g NaCl using vortex VWR international (Barcelona, Spain) for 2 minutes. The extract was centrifuged at 3000 rpm (Eppernord AG 22331, Hamburg, Germany) for 10 minutes, and the supernatant was treated and mixed using vortex for 2 minutes with 150 mg C_{18} and 900 mg $MgSO_4$. The samples were centrifuged at 3000 rpm (Eppernord AG 22331, Hamburg, Germany) for 10 minutes and the supernatant was completely dried using a continuous flow of nitrogen (Turbovap LV, Zymark Runcorn, UK). Finally, the sample was suspended in 1 mL of water : acetonitrile (90 : 10 v/v), filtered through a 0.22 µm nylon filter (Phenomenex, California, Stati Uniti) and stored for chromatographic analysis.

An Agilent 1200-LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a vacuum degasser, auto-sampler, and binary pump was used for the chromatographic determination. Chromatography separation was performed using a Gemini (C_{18} 50 × 2 mm, 100 A, 3 μm) column (Phenomenex, Torrance, California, UK). Mobile phases consisting of 1% acetic acid as solvent system A and acetonitrile as solvent system B and the following gradient elution were used: 0 min, 0.8% B; 5.5 min, 6.8% B; 16 min, 20% B; 20 min, 25% B; 25 min, 35% B; 29 min, 100% B; 32 min, 100% B; 34 min, 0.8% B; 36 min, 0.8% B. The column was equilibrated for 3 min prior to every analysis. The sample volume injected was 20 μL and the flow rate used was 0.8 mL/min.

MS analyses were carried out using a 6540 Agilent Ultra- High-Definition Accurate-Mass q-TOF-MS coupled to the HPLC, equipped with an Agilent Dual Jet Stream electrospray ionization (Dual AJS ESI) interface in negative ionization mode with the following conditions: drying gas flow (N2), 12.0 L/ min; nebulizer pressure, 50 psi; gas drying temperature, 370 °C; capillary voltage, 3500 V; fragmentor voltage, and scan ranges were 3500 V and m/z 50–1500, respectively. Automatic MS/MS experiments were carried out using the following collision energy values: m/z 100, 30 eV; m/z 500, 35 eV; m/z 1000, 40 eV; and m/z 1500, 45 eV. Integration and data elaboration were performed using Mass Hunter Work station software (Agilent Technologies).

Statistical analysis

Analysis of variance was carried out to assess whether the different experiments led to statistically different results for those variables evaluated. Statistical analysis of data was performed using the Tukey's test. Results are

expressed as mean values ± s.e. mean. The chosen significance level was $P < 0.01$.

Results and discussion

Antifungal activity, MIC–MFC of freeze-dried fermented whey obtained by fermentation with different strains of Lactobacillus plantarum correlation with organic compounds

The use of LAB in the food industry is widespread because of its positive contribution to the flavour, texture and nutritional value in food products, besides its natural antimicrobial properties that extend their shelf life. The proteolytic systems of LAB, which include proteinases and peptidases, are a considerable means to produce peptides and amino acids from milk proteins during fermentation (Ström *et al.* 2002, Lavermicocca *et al.* 2002, Lavermicocca *et al.* 2003). Proteinases are located in the cell wall and belong to the serine protease group, and they are able to make peptides starting caseins. Peptidases can be both extra and intra-cellular and are responsible for cleaving large peptides into small peptides (Kenny *et al.* 2003). Probably, secondary metabolites produced during fermentation by LAB, including organic acids, supply an unfavorable acid environment for the growth of pathogenic and deteriorating microorganisms. Acids are generally responsible for the antimicrobial effect due to their interfering nature with the maintenance of the potential cell membrane, reducing intracellular pH, inhibiting active transport and a variety of metabolic functions (Taylor *et al.* 2014).

In Table 13, the results associated with the antifungal activity of freeze-dried fermented whey by different strains of *L. plantarum* against 9 strains of mycotoxigenic *Aspergillus*, *Penicillium* and *Fusarium* strains are plotted. The

results highlight that whey fermentedby different strains of *L. plantarum* do not have activity against mycotoxigenic *Penicillium* and *Aspergillus* strains at different fermentation times (24, 48, 72 h). As regards *Fusarium* genera, an antifungal activity of fermented whey by *L. plantarum* 220 and 221 was observed. In particular, a clearing zone of 8–10 mm against mycotoxigenic fungi *F. graminearum, F. verticilliodes* at 24 hours of fermentation time, respectively were exhibited. The highest antifungal activity was observed for whey fermented by *L. plantarum* 220 against the *Fusarium* strains.

Whey fermented by *L. plantarum* 748 showed an antifungal activity on *F. verticilliodes* exhibiting a clearing zone of 8 mmat 24 hours of fermentation time. The antifungal activity increased with fermentation time (48, 72 h), showing a clearing zone of 10 mm on *F. moniliformis, F. graminearum* and *F. verticilliodes* at 72 h of fermentation. In Table 14, it is possible to observe the results related to the Minimum inhibitory Concentration and the Minimum Fungicidal Concentration (MIC–MFC) of fermentedwhey by different strains of *L. plantarum*at 72 h of fermentation time. In particular, as regards the *Fusarium* generum, the MIC ranged from 31.3 to 250 g/L, whereas the MFC data ranged from 125 to 250 g/L for whey fermented by three strains of *L. plantarum* (220, 221, 748). For *Penicillium* strains, the MIC ranged from 62.5 to 250 g/L, while the MFC presented the value of 250 g/L necessary to obtain the minimum fungicidal concentration. Whey fermented by *L. plantarum* 748 evidenced the slightest antifungal activity, showing a MIC of 250 g/L. The antifungal activity of whey fermented by LAB has not yet been extensively studied. In particular, Gamba *et al.* 2015 investigated the antifungal activity of permeate fermented whey by Kefir grain against *A. parasiticus*, acommon fungal contaminant in food, which has the capability to produce aflatoxin B1. A fungicidal effect was obtained with 65% v/v of cell free supernatant in the

culture medium at pH 4.55. In these conditions, aflatoxin production was not detected.

Tulini *et al.* 2016 investigated the role of LAB strains isolated from different dairy products (milk and cheese samples), in order to evaluate the correlation with the antimicrobial activity. Among the strains studied, *L. plantarum FT723* presented the most antifungal activity able to inhibit *P. expansum* in a modified MRS agar and fermented milk model. LAB couldproduce several antimicrobial compounds which make these bacteria interesting for food bio-preservation.

Table 13. Antifungal activity of freeze-dried whey obtained from fermentation with three different *Lactobacillus* in three different times of fermentation (24, 48, 72h) using the antimicrobial assay on solid medium of PDA. Clearing zone (+) corresponding to 8 mm, (++) corresponding to 10 mm.

Fungi	***L.plantarum 220***			***L.plantarum 221***			***L.plantarum 748***		
	24h	**48h**	**72h**	**24h**	**48h**	**72h**	**24h**	**48h**	**72h**
***P. camemberti* CECT 2267**	-	-	-	-	-	-	-	-	-
***P. expansum* CECT 2278**	-	-	-	-	-	-	-	-	-
***P. roqueforti* CECT 2905**	-	-	-	-	-	-	-	-	-
***A. parasiticus* CECT 2681**	-	-	-	-	-	-	-	-	-
***A. flavus* ITEM 8111**	-	-	-	-	-	-	-	-	-
***A. niger* CECT 2088**	-	-	-	-	-	-	-	-	-
***F. moniliformis* CECT 2982**	-	++	++	-	++	++	-	++	++
***F. verticilliodes* CECT 20926**	++	++	++	++	++	++	-	++	++
***F. graminearum* CECT 20490**	+	++	++	+	++	++	+	++	++

Table 14. Minimum inhibitory concentration and minimum fungicidal concentration expressed in g/L (MIC-MFC) evidenced by freeze-dried whey obtained by fermentation with three different *Lactobacillus* at 72h of fermentation time, on several mycotoxigenic fungi; (a= g/L).

Fungi	***L.plantarum 220***		***L.plantarum 221***		***L.plantarum 748***	
	MIC[a]	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**
P. camemberti	62.5	250	125	125	nd	nd
P. expansum	62.5	250	62.5	250	250	250
P. roqueforti	125	125	125	125	125	125

A. parasiticus	250	250	250	250	250	125
A. flavus	250	250	250	250	250	nd
A. niger	250	250	nd	nd	nd	nd
F. moniliformis	62.5	125	125	250	250	250
F. verticilliodes	62.5	62.5	125	125	250	250
F. graminearum	31.3	250	62.5	250	125	125

Antifungal activity, MIC–MFC of sweet fermented whey obtained by fermentation with different strains of Lactobacillus plantarum

The qualitative antifungal activity of SFW against the tested fungi is detailed in Table 15. Sweet whey fermented by four different LABs were active against *Aspergillus*, *Penicillium* and *Fusarium* genera. In the analysis of data, it was observed that sweet whey fermented by *L. plantarum* 220, *L. plantarum* 221, *L. plantarum* 223 were more active than sweet whey fermented by *L. plantarum* 748. The most satisfying inhibitory effect was shown against *A. flavus, A. carbonarius, P. verrucosum, P. nordicum, P. pinophilum* and all the *Fusarium* species. The diameters of the inhibition zones toward the pathogenic strains varied between 8.0 and 10.0 mm. The lowest inhibitory activity was obtained against *A. lacticoffeatus* from all four type of bacteria utilized in the fermentation process. In figure 6 is reported an example of antifungal activity in the solid medium.

Table 15. Antifungal activity of sweet fermented whey by four different strains of *L. plantarum* using the antimicrobial assay on solid medium of PDA. The diameters of the inhibition zones toward the pathogenic strains varied between 8 and 10 mm: clearing zone (+) corresponding to 8 mm, (++) corresponding to 9mm, (+++) corresponding to 10 mm.

Fungi	**CECT/ ITEM**	***SFW CECT 220***	***SFW CECT 221***	***SFW CECT 223***	***SFW CECT 748***
Aspergillus parasiticus	**CECT 2681**	++	+	++	+
Aspergillus steynii	**CECT 20510**	++	+	++	-

Aspergillus flavus	ITEM 8111	+++	++	+++	++
Aspergillus carbonarius	ITEM 5010	+++	++	+++	+
Aspergillus niger	CECT 2088	+	+	+	-
Aspergillus brasiliensis	CECT 2574	++	+	++	+
Aspergillus sclerotioniger	CECT 20583	++	+	+	-
Aspergillus tubingensis	CECT 50543	++	++	++	+
Aspergillus tubingensis	CECT 50544	++	+	++	-
Aspergillus lacticoffeatus	CECT 20581	-	-	+	-
Penicillium camamberti	CECT 2267	+++	++	+++	-
Penicillium roqueforti	CECT 2905	+	+	+	-
Penicillium verrucosum	VTT D-01847	+++	+++	+++	++
Penicillium digitatum	CECT 2954	+++	++	+++	-
Penicillium nordicum	CECT 2320	+++	+++	+++	-
Penicillium commune	CECT 20767	+++	++	+++	+
Penicillium expansum	CECT 2278	+++	+++	+++	++
Penicillium brevicompactum	CECT 2316	+++	+++	+++	++
Penicillium solitum	CECT 20818	+++	++	+++	+
Penicillium pinophilum	CECT 2912	+++	++	+++	+
Fusarium moniliforme	CECT 2987	+++	+++	+++	-
Fusarium moniliforme	CECT 2152	+++	+++	+++	-
Fusarium graminearum	ITEM 6415	+++	+++	+++	-
Fusarium proliferatum	ITEM 12072	+++	+++	+++	++
Fusarium verticillioides	ITEM 12052	+++	++	+++	+
Fusarium verticillioides	ITEM 12043	+++	+++	+++	+
Fusarium verticillioides	ITEM 12044	+++	+++	+++	+
Fusarium sporotrichioides	ITEM 12168	+++	+++	+++	+
Fusarium langsethiae	ITEM 11031	+++	+++	+++	+
Fusarium poae	ITEM 9151	+++	+++	+++	++

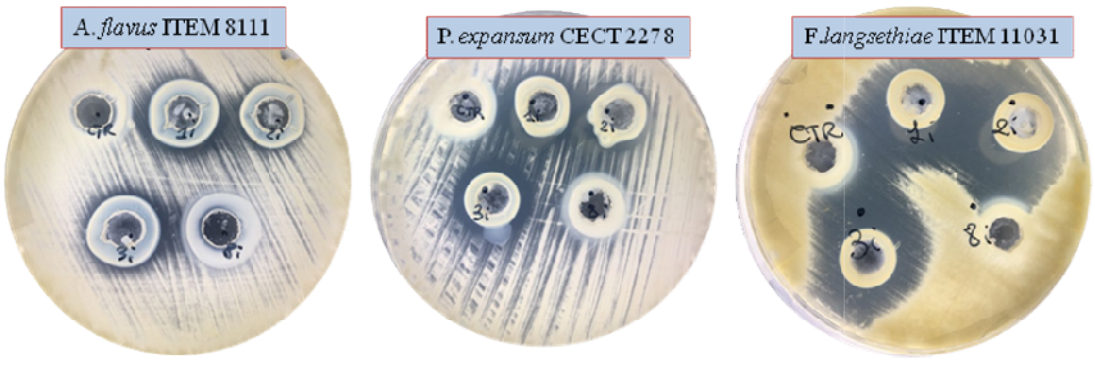


Figure 6. An example of antifungal activity of sweet fermented whey in the solid medium on three different strain of mycotoxigenic fungi.

The antifungal activity of goat's fermented whey in liquid medium against the tested fungi allowed us to determine the Minimum Inhibitory Concentration and the Minimum Fungicidal Concentration (MIC–MFC). In picture 4 is reported an example of antifungal activity in the liquid medium. The values of MIC–MFC of goat's whey fermented by different strains of *L. plantarum* at 72 hours of fermentation time was contained between 1.95 and 250 g/L and are detailed in table 16. The value of MIC for *Aspergillus* and *Fusarium* genera was contained between 1.95 and 62.5 g/L whereas for *Penicillium* genera was contained between 3.9 and 125 g/L. The value of MFC was included in a range of: 15.6-250 g/L for *Aspergillus*, 62.5-250 g/L for *Penicillium* and 3.9-250 g/L for *Fusarium*. The lowest MIC values (1.95 g/L) was shown from goat's whey fermented by *L. plantarum* 223 against *A. steynii, F. moniliforme, F. verticillioides, F. sporotrichioides, F. langsethiae, F. poae* species and whey fermented by *L.plantarum 220* against *A.stenyii, F. langsethiae, F. poae* species. Less active SFW show higher MIC and MFC values (250 g/L) as reported by sweet whey fermented by *L. plantarum* 748 against *Penicillium* and *Aspergillus* genera. Particularly interesting were the results of sweet whey fermented by *L. plantarum* 220, 221, 223 against *P. pinophilum*. For the *Aspergillus* genera, satisfying inhibitory effect were obtained against *A. steynii* for sweet whey fermented by *L. plantarum* 220 and against *A. cabonarius, A. lacticoffeatus* for goat's whey fermented by *L. plantarum* 223. Regarding *Fusarium* genera, all species have shown a good inhibition effect after fermentation process using *L. plantarum* 220 and *L. plantarum* 223 strains.

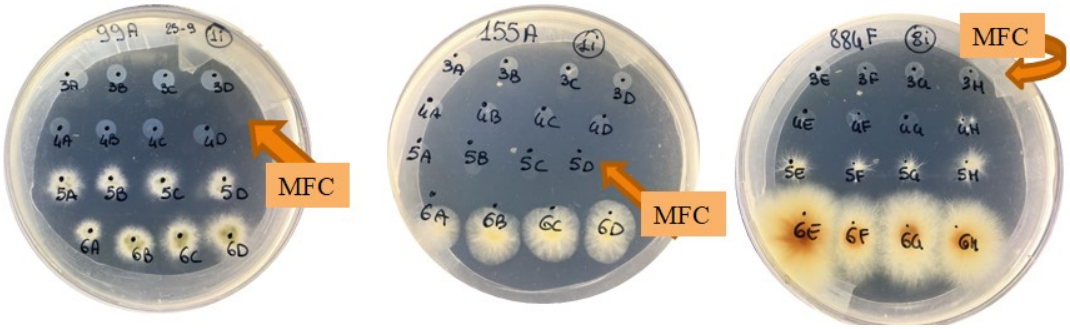


Figure 7. An example of antifungal activity of sweet fermented whey in the liquid medium on mycotoxigenic fungi.

Table 16. Minimum inhibitory concentration and minimum fungicidal concentration (MIC–MFC) obtained by sweet whey after fermentation process with four different *Lactobacillus* at 72 h of fermentation time, on 28 mycotoxigenic fungi; (a = g/L)

Fungi	CECT/ ITEM	MIC g/L	MFC g/L	MIC g/L	MFC g/L	MIC g/L	MFC g/L	MIC g/L	MFC g/L
		SFW CECT 220		*SFW CECT 221*		*SFW CECT 223*		*SFW CECT 748*	
Aspergillus parasiticus	**CECT 2681**	31.2	125	15,6	125	62.5	250	125	250
Aspergillus steynii	**CECT 20510**	1.95	62.5	7.8	62.5	1.95	125	31.2	250
Aspergillus flavus	**ITEM 8111**	31.2	62.5	31.2	62.5	7.8	62.5	62.5	250
Aspergillus carbonarius	**ITEM 5010**	31.2	31.2	31.2	62.5	3.9	31.2	62.5	125
Aspergillus niger	**CECT 2088**	31.2	31.2	31.2	31.2	7.8	15.6	62.5	125
Aspergillus brasiliensis	**CECT 2574**	31.2	125	31.2	125	15.6	31.2	62.5	125
spergillus sclerotioniger	**CECT 20583**	31.2	62.5	31.2	62.5	7.8	31.2	62.5	125
Aspergillus tubingensis	**CECT 50543**	31.2	31.2	31.2	31.2	7.8	15.6	62.5	62.5
Aspergillus tubingensis	**CECT 50544**	31.2	31.2	31.2	62.5	7.8	15.6	62.5	125
Aspergillus lacticoffeatus	**CECT 20581**	31.2	62.5	31.2	62.5	3.9	15.6	62.5	250
Penicillium camemberti	**CECT 2267**	62.5	250	125	250	15,6	-	31.2	-
Penicillium roqueforti	**CECT 2905**	62.5	-	62.5	-	62.5	-	125	-
Penicillium verrucosum	**VTT D-01847**	15.6	62.5	15.6	125	7.8	125	31.2	125
Penicillium digitatum	**CECT 2954**	62.5	-	62.5	-	125	-	125	-
Penicillium commune	**CECT 2320**	62.5	125	31.2	250	31.2	125	62.5	125
Penicillium solitum	**CECT 20767**	31.2	125	31.2	125	31.2	125	62.5	250
Penicillium nordicum	**CECT 20818**	7.8	62.5	31.2	62.5	62.5	125	62.5	250
Penicillun expansum	**CECT 2278**	7.8	62.5	7.8	62.5	3.9	62.5	31.5	250
Penicillium brevicompactum	**CECT 2316**	7.8	62.5	7.8	62.5	7.8	62.5	31.2	250
Penicillium pinophilum	**CECT 2912**	7.8	62.5	7.8	62.5	3.9	62.5	15.6	250

Fusarium moniliforme	**CECT 2987**	7.8	125	7.8	125	3.9	15.6	31.2	125
Fusarium moniliforme	**CECT 2152**	7.8	31.2	7.8	125	1.95	62.5	31.2	62.5
Fusarium graminearum	**ITEM 6415**	7.8	31.2	7.8	62.5	3.9	62.5	31.2	62.5
Fusarium proliferatum	**ITEM 12072**	15.6	31.2	15.6	31.2	3.9	7.8	31.2	62.5
Fusarium verticillioides	**ITEM 12052**	7.8	62.5	7.8	250	1.95	7.8	31.2	125
Fusarium verticillioides	**ITEM 12043**	7.8	62.5	15.6	62,5	3.9	31.2	31.2	250
Fusarium verticillioides	**ITEM 12044**	7.8	62.5	7.8	31.2	3.9	7.8	31.2	125
Fusarium sporotrichioides	**ITEM 12168**	7.8	31.2	15.6	31.2	1.95	15.6	31.2	62,5
Fusarium langsethiae	**ITEM 11031**	1.95	3.9	7.8	15.6	1.95	3.9	7.8	15.6
Fusarium poae	**ITEM 9151**	1.95	31.2	7.8	15.6	1.95	3.9	7.8	62.5

The antifungal activity of goat's whey fermented by LAB has not yet been extensively studied. Londero *et al.* 2014 have studied the antifungal activity of whey fermented with kefir grains for 24 h at 20 °C. After 24 h of fermentation, the kefir grains were separated from the fermentation products by filtration through a plastic sieve. The grains showed a high percentage of inhibition of the conidium germination (≥ 70%) in the species of *Aspergillus*, *Penicillium* and *Fusarium*. Also Gamba *et al.* 2016 have studied the antifungal capacity of cell-free-supernatants from whey permeate obtained after fermentation of kefir grains against *Fusarium graminearum* growth and the production of zearalenone (ZEA). It was observed that, the fermented supernatants of pH 3-5 produced the highest percentages of inhibition of the conidial germination. Alkalinization led to the gradual loss of antifungal activity. Results shown that the supernatants of kefir had antifungal activity against the strains of *F. graminearum* investigated and inhibited the production of mycotoxins.

Several potential health or nutritional benefits could be possible using some species of LAB on fermentation process in dairy products (Borresen *et al.* 2012, Swain *et al.* 2016). The production of antimicrobial substances by the use of starter cultures, for instance, LABs led to rapid acidification of the raw

material, providing an unfavourable environment for microorganism growth (Soro-Yao *et al.* 2014). In a recent study conducted by Juodeikiene *et al.* 2018, was investigated the treatment with LABs in malting wheat grains to finding a strategy for reduction of *Fusarium* mycotoxin. LABs strains (*Lactobacillus sakei KTU05-6, Pediococcus acidilactici KTU05-7, and Pediococcus pentosaceus KTU05-8, KTU05-09, KTU05-10*) have revealed a broad spectrum of antimicrobial activity against *Fusarium culmorum* and *Fusarium poae*. The treatment of wheat grains with permeate previously fermented with *P. acidilactici KTU05-7* and *P. pentosaceus KTU05-10* strains resulted in increased germination of wheat grains by 9.5 and 7.9% respectively comparing with grains that were not pre-treated. Also Fernandez *et al.* 2017 have screened 88 strains of food-grade bacteria for their inhibition effect on four spoilage moulds commonly isolated from cheese. Strains of *Propionibacterium* and *Lactobacillus* were the most active. Particularly, *Lactobacillus rhamnosus A238* alone or in combination with *Bifidobacterium animalis subsp. lactis A026* was able to inhibit mold growth for at least 21 days at 6°C, due probably to the production of secondary metabolites or competition for nutrients. The use of different strains of LAB in the fermentation process of whey allowed to obtain good results in antifungal activity tests. In fact, as demonstrated that, fermentation process using LAB impacted on the phenolic profile (Kwaw *et al.* 2018, Sánchez-Maldonado *et al.* 2011).

Identification of phenolic compounds

Antimicrobial activity of LAB is associated with the production of different active molecules such as peptides, antifungal compounds and organic acids (Kralik *et al.* 2018, Rodríguezet *et al.* 2009). However, there are other preservation mechanisms, such as bacteriocins production, that are suspected of being involved in the inactivation or inhibition of the growth of

other associated species of pathogens. A large number of bacteriocins produced by LABs have been identified, although their potential application as bio-preservatives has not been fully developed. (Cheikhyoussef *et al.* 2009, Tremonte *et al.* 2017, Dinev *et al.* 2018). The analysis of total phenolic compounds connected with freeze-dried fermented whey after 72 h of treatment led to the identification of ten different compounds that are shown in Table 16. In particular, in the FDW fermented by *L. plantarum* 220, the following were detected: hydroxibenzoic acid, sinapic acid, benzoic acid and DL-3-phenillactic acid, whereas in the FDW fermented by *L. plantarum* 221, ferulic acid, hydroxibenzoic acid, sinapic acid, benzoic acid and DL-3-phenillactic acid were discovered. Finally, in the FDW fermented by *L. plantarum* 748, caffeic acid, p-coumaric acid, salicilic acid, hydrocinnamic acid, DL-3-phenillactic and 1,2-dihydroxibenzene were detected.

Benzoic acid and sodium benzoate are antifungal agents and they are employed in industries to contribute to preservationof foods. Strains of *L. acidophilus*, *L. casei* and *L.s helveticus* used for milk fermentation can produce benzoic acid infermented milk (Garmiene *et al.* 2010, Schnürer, *et al.* 2005). Recently, Kwaw *et al.* 2018 investigated the effect of LAB strains on the phenolic profile and antioxidant activities of mulberry juice. The fermentation process using *L. plantarum* demonstrated that LABs impacted on the phenolic profile. In addition, Valerio *et al.* 2009 studied the organic acids in the LAB from semolina in order to find a correlation with the antifungal activity of bacterial strains. All strains isolated acidified the medium, leading to pH values ranging from 3.05 to 3.85, and they have the capability to produce organic acids. The inhibitory effect of pure lactic and acetic acids was tested. Results indicated an inhibitory effect of the medium composed of both organic acids of 99.9%, 75.5% and 97.4% inhibition against *A. niger*, *P. roqueforti*and *E. fibuliger*, respectively. Phenolic compounds

showed microbicidal properties against different microorganisms (Gañan *et al.* 2009).

The phenolic acids studied in the SFW are summarized in Table 18 including molecular formula, experimental and calculated m/z, and fragments. The analysis of phenolic compounds connected with SFW after 72 h of treatment led to the identification of the predominant one: DL 3-phenillactic (PLA). This phenolic compound was found in all SFW by different strains of *L. plantarum*. The results were expressed as mg/L of PLA compared to the control. The quantitative of PLA obtained in SFW by *L. plantarum* strains were: 1.18±0.03, 0.68±0.04, 1.21±0.02 and 0.003±0.01 for *L. platarum* 220, 221, 223 and 748, respectively.

These phenolic compounds, present in all whey fermented by different strains of *L. plantarum*, have been widely studied by researchers for their fungal inhibition capability (Ansari *et al.* 2013, Carvalho *et al.* 2018, Alves *et al.* 2014). PLA is an important phenolic acid, produced during microbial fermentation using the strains of LAB. Due to the metabolism of phenylalanine in LAB, the latter was transaminated to phenylpyruvic acid (PPA) and PPA further reduced to PLA (Vermeulen *et al.* 2006; Li *et al.*2007, Chaudhari, *et al.* 2016). The fermentation production of PLA with different microorganisms has been investigated by researchers in the past years. Previous studies have reported that PLA is an antimicrobial compound with a wide activity spectrum against some yeast and moulds including mycotoxigenic species such as *Aspergillus* and *Penicillium* (Dieuleveux *et al.* 1998, Valerio *et al.* 2004 Lavermicocca, Valerio, & Visconti, 2003; Yoo *et al* 2010). A wide variety of LAB has been screened by researchers for their antifungal potential and their capability to produce PLA (Cortés-Zavaleta *et al.* 2014, Gerez *et al.* 2009, Crowley *et al.* 2013, Gerez *et al.* 2013).

Table 17. Phenolic compounds identified in freeze-dried whey obtained from 24, 48, 72h of fermentation with three different *Lactobacillus* by LC-qTOF-MS".

Compound	Molecular formula	*m/z* Calculated $(M-H)^-$	*m/z* Detected $(M-H)^-$	Fragments	*L. plantarum 220*	*L. plantarum 221*	*L. plantarum 748*
Caffeic acid	$C_9H_8O_4$	790.1629	179.0323	135.0447/89.0404/107.0506	-	-	+
p-Coumaric acid	$C_9H_8O_3$	163.1611	163.0378	119.0497/93.0349	-	-	+
Ferulic acid	$C_{10}H_{10}O_4$	193.1828	193.0475	134.0364/ 178.0251/ 106.0428/ 78.9589/ 152.8921	-	+	-
Hydroxibenzoic acid	$C_7H_6O_3$	137.1233	137.0233	93.0351/ 65.0408	+	+	-
Sallicilic acid	$C_7H_6O_3$	137.1211	137.0235	93.0351/ 65.0405	-	-	+
Hydrocinnamic acid	C_9H10O_2	149.1711	149.0593	63.0047/ 78.9609/ 84.9856/ 96.9594/ 133.8511/ 128.5397/ 58.3563	-	-	+
Sinapic acid	$C_{11}H_{12}O_5$	223.2156	223.0568	193.0118/ 93.0350/ 121.0292/ 149.0232/ 163.0393/ 208.0348/ 135.0446/ 126.9043/ 89.0244	+	+	-
Benzoic acid	$C_7H_6O_2$	121.1212	121.5028	87.9241/ 64.6394/ 97.9436/ 67.5325/ 105.8440/ 78.9786/ 43.8011/ 50.4725	+	+	-
DL-3-phenyllactic acid	$C_9H_{10}O_3$	165.1750	165.0532	103.0556/ 72.9940/ 119.0500/ 147.0447/ 77.0403/ 91.0563/ 44.9996	+	+	+
1-2 dihydroxybenzene	$C_6H_6O_2$	109.1145	109.0288	108.0218/ 65.0042/ 91.0195/ 53.0407/ 81.0349	-	-	+

Valerio *et al.* 2004 reported that the organic acids: PLA and 4-hydroxyphenyllactic acid (HPLA), could be produced by a wide range of LAB species such as *Lactobacillus, Enterococcus, Weissella*, and *Leuconostoc.* It is presumed that the behaviour of the antifungal activity of whey was caused by acidic compounds like PLA or organic acids rather than proteins or peptides molecules (Martins *et al.* 2012, O'connell *et al.* 2001, Li *et al.* 2015, Ryan *et al.* 2011, Mu *et al.* 2009).

Rodriguez *et al.* 2011 have studied the ability to produce PLA from five different LABs. The highest amount of PLA was produced by *L. plantarum* CECT 221, the same strain used in our study. A GC-FID analysis of the metabolites obtained in the phenylalanine metabolism were showed the

presence of small amounts of benzaldehyde and phenylacetaldehyde. Exhausted broths containing mixtures of PLA and other organic acids resulted to be an effective antimicrobial against the pathogen *Salmonella* reducing his growth in 63.2% in 24 h of incubation.

Lavermicocca *et al.* 2000 have evaluated the antifungal activity of PLA obtained during fermentation of wheat flour by *L. plantarum* 21B strain. The high concentration of the phenolic compound in the bacterial culture filtrate, allowed to inhibit all fungi tested belonging to the *Aspergillus*, *Penicillium* genera at a concentration of 50 mg/mL. For *P. roqueforti IBT18687* and *P. corylophilum IBT6978* the inhibitory effect was obtained at a concentration of 166 mg/mL. Also in our study, SFW did not show adequate inhibitory results against *P. roqueforti* strain. A subsequent study conducted by Lavermicocca *et al.* 2003 reported that fermentation process with *Saccharomyces cerevisiae* and the PLA-producing LAB strain, *L. plantarum* 21B, delayed for 7 days the fungal growth in bread.

So, this interesting organic acid provides new perspectives for the possibility of using this natural antimicrobial compound as a fungicide or as food preservative, feed additive, pharmaceutical agent and therefore, has potential applications in chemical, pharmaceutical, biotechnological and food industries.

Table 18. Phenolic compounds identified in sweet whey obtained from 72h fermentation with different *Lactobacillus* by LC-qTOF-MS".

Compounds	Molecular formula	m/z calculated (M-H)+	m/z experimental (M-H)+	Fragments	*L. plantarum 220*	*L. plantarum 221*	*L. plantarum 223*	*L. plantarum 748*
Gallic	$C_7H_6O_5$	169.12	169.0117	125.0242-79.0194-51.0248-69.0353-97.0305-107.0139	+			
Protocatechuic	$C_7H_6O_4$	153.12	153.0172	109.0295-68.9962-91.0194-60.9879-53.0404			+	
Chlorogenic	$C_{16}H_{15}O_9$	353.31	353.0653	191.0543-85.0301-93.0349-44.999-59.0146-127.0397	+			+
Caffeic	$C_9H_8O_4$	179.16	179.0323	135.0447-89.0404-107.0506				
Syringic	$C_9H_{10}O_5$	197.17	197.0427	123.0083-95.0138-182.0220-78.0121-166.996-138.0317-89.0034-51.0264-61.9894-103.9732-147.8891	+			
Vanillin	$C_8H_8O_3$	151.13	151.0381	136.0160-92.0270-108.0217-44.9990-58.9730-68.9974-76.9614-146.7437		+		
p-Coumaric	$C_9H_8O_3$	163.16	163.0378	119.0497-93.0349		+		
Ferulic	$C_{10}H_{10}O_4$	193.18	193.0475	134.0364-178.0251-106.0428-78.9589-152.8921				
Hydroxibenzoic	$C_7H_6O_3$	137.12	137.0233	93.0351-65.0408	+	+	+	
Vanillic	$C_8H_8O_4$	167.15	167.029	108.0218-152.0107-91.0196-65.0039-44.9987-123.0458-134.9553-55.1623-75.3574-112.8742				
Sallicilic	$C_7H_6O_3$	137.12	137.0235	93.0351-65.0405	+	+	+	+
Hydrocinnamic	C_9H10O_2	149.17	149.059	63.0047-78.9609-84.9856-96.9594-133.8511-128.5397-58.3563				+
Sinapic	$C_{11}H_{12}O_5$	223.21	223.0568	193.0118-93.0350-121.0292-149.0232-163,0393-208.0348-135.0446-126.9043-89.0244	+			+
Benzoic	$C_7H_6O_2$	121.12	121.0284	87.9241-64.6394-97.9436-67.5325-105.8440-78.9786-43.8011-50.4725				
DL-3-phenyllactic	$C_9H_{10}O_3$	165.17	165.0532	103.0556-72.9940-119.0500-147.0447-77.0403-91.0563-44.9996	+	+	+	+
1-2 dihydroxybenzene	$C_6H_6O_2$	109.11	109.0288	108.0218-65.0042-91.0195-53.0407-81.0349		+		+
3,4-dihydroxyhydrocinnamic	$C_9H_{10}O_4$	181.17	181.0478	113.3938-83.9225-57.3755-45.2726-94.4798-75.3451				

References:

- Alves, C. T., Ferreira, I. C., Barros, L., Silva, S., Azeredo, J., & Henriques, M. (2014). Antifungal activity of phenolic compounds identified in flowers from North Eastern Portugal against Candida species. *Future microbiology*, 9(2), 139-146.
- Ansari, M. A., Anurag, A., Fatima, Z., & Hameed, S. (2013). *Natural phenolic compounds: a potential antifungal agent*. *Microb*, 189-95.
- Brosnan, B., Coffey, A., Arendt, E. K., & Furey, A. (2014). The QuEChERS approach in a novel application for the identification of antifungal compounds produced by lactic acid bacteria cultures. *Talanta*, 129, 364-373.
- C Borresen, E., J Henderson, A., Kumar, A., L Weir, T., & P Ryan, E. (2012). Fermented foods: patented approaches and formulations for nutritional supplementation and health promotion. *Recent patents on food, nutrition & agriculture*, 4(2), 134-140.
- Carvalho, R. S., Carollo, C. A., de Magalhaes, J. C., Palumbo, J. M. C., Boaretto, A. G., e Sá, I. N., & Ferreira, J. M. S. (2018). Antibacterial and antifungal activities of phenolic compound-enriched ethyl acetate fraction from Cochlospermum regium (mart. Et. Schr.) Pilger roots: Mechanisms of action and synergism with tannin and gallic acid. *South African Journal of Botany*, 114, 181-187.
- Chaudhari, S. S., & Gokhale, D. V. (2016). Phenyllactic acid: A potential antimicrobial compound in lactic acid bacteria. *J Bacteriol Mycol Open Access*, 2(5), 00037.
- Cheikhyoussef, A., Pogori, N., Chen, H., Tian, F., Chen, W., Tang, J., & Zhang, H. (2009). Antimicrobial activity and partial characterization of bacteriocin-like inhibitory substances (BLIS) produced by Bifidobacterium infantis BCRC 14602. *Food Control*, 20(6), 553-559.
- Cortés-Zavaleta, O., López-Malo, A., Hernández-Mendoza, A., & García, H. S. (2014). Antifungal activity of lactobacilli and its relationship with 3-phenyllactic acid production. *International journal of food microbiology*, *173*, 30-35.
- Crowley, S., Mahony, J., & van Sinderen, D. (2013). Current perspectives on antifungal lactic acid bacteria as natural bio-preservatives. *Trends in Food Science & Technology*, *33*(2), 93-109.
- Dieuleveux, V., Lemarinier, S., & Gueguen, M. (1998). Antimicrobial spectrum and target site of D-3-phenyllactic acid. *International Journal of Food Microbiology*, *40*(3), 177-183.

- Dinev, T., Beev, G., Tzanova, M., Denev, S., Dermendzhieva, D., & Stoyanova, A. (2018). Antimicrobial activity of Lactobacillus plantarum against pathogenic and food spoilage microorganisms: a review. *Bulgarian Journal of Veterinary Medicine*, 21(3).
- Fernandez, B., Vimont, A., Desfossés-Foucault, É., Daga, M., Arora, G., & Fliss, I. (2017). Antifungal activity of lactic and propionic acid bacteria and their potential as protective culture in cottage cheese. *Food Control*, 78, 350-356.
- Fothergill, A. W. (2012). Antifungal susceptibility testing: clinical laboratory and standards institute (CLSI) methods. In *Interactions of yeasts, moulds, and antifungal agents* (pp. 65-74). *Humana Press.*
- Gamba, R. R., De Antoni, G., & Peláez, A. L. (2016). Whey permeate fermented with kefir grains shows antifungal effect against *Fusarium* graminearum. *Journal of Dairy Research*, 83(2), 249-255.
- Gamba, R. R., Ni Colo, C., Correa, M., Astoreca, A. L., Alconada Magliano, T. M., De Antoni, G., & Leon Pelaez, A. (2015). Antifungal activity against *Aspergillus* parasiticus of supernatants from whey permeates fermented with kéfir grains.
- Gañan, M., Martínez-Rodríguez, A. J., & Carrascosa, A. V. (2009). Antimicrobial activity of phenolic compounds of wine against Campylobacter jejuni. *Food Control*, 20(8), 739-742.
- Garmiene, G., Salomskiene, J., Jasutiene, I., Macioniene, I., & Miliauskiene, I. (2010). Production of benzoic acid by lactic acid bacteria from Lactobacillus, Lactococcus and Streptococcus genera in milk. *Milchwissenschaft*, 65(3), 295-298.
- Gerez, C. L., Torino, M. I., Rollán, G., & De Valdez, G. F. (2009). Prevention of bread mould spoilage by using lactic acid bacteria with antifungal properties. *Food control*, *20*(2), 144-148.
- Gerez, C. L., Torres, M. J., De Valdez, G. F., & Rollán, G. (2013). Control of spoilage fungi by lactic acid bacteria. *Biological Control*, *64*(3), 231-237.
- Juodeikiene, G., Bartkiene, E., Cernauskas, D., Cizeikiene, D., Zadeike, D., Lele, V., & Bartkevics, V. (2018). Antifungal activity of lactic acid bacteria and their application for *Fusarium* mycotoxin reduction in malting wheat grains. *LWT-Food Science and Technology*, 89, 307-314.
- Kenny, O., FitzGerald, R. J., O'Cuinn, G., Beresford, T., & Jordan, K. (2003). Growth phase and growth medium effects on the peptidase activities of Lactobacillus helveticus. *International dairy journal*, 13(7), 509-516.

- Kralik, P., Babak, V., & Dziedzinska, R. (2018). The Impact of the Antimicrobial Compounds Produced by Lactic Acid Bacteria on the Growth Performance of Mycobacterium avium subsp. paratuberculosis. *Frontiers in microbiology*, 9, 638.
- Kwaw, E., Ma, Y., Tchabo, W., Apaliya, M. T., Wu, M., Sackey, A. S., ... & Tahir, H. E. (2018). Effect of lactobacillus strains on phenolic profile, color attributes and antioxidant activities of lactic-acid-fermented mulberry juice. *Food chemistry*, 250, 148-154.
- Lavermicocca, P., Valerio, F., & Visconti, A. (2003). Antifungal activity of phenyllactic acid against molds isolated from bakery products. *Applied and Environmental Microbiology*, 69(1), 634-640.
- Lavermicocca, P., Valerio, F., Evidente, A., Lazzaroni, S., Corsetti, A., & Gobbetti, M. (2000). Purification and characterization of novel antifungal compounds from the sourdough Lactobacillus plantarum strain 21B. *Applied and Environmental Microbiology*, 66(9), 4084-4090.
- Li, X., Jiang, B., & Pan, B. (2007). Biotransformation of phenylpyruvic acid to phenyllactic acid by growing and resting cells of a *Lactobacillus sp*. *Biotechnology Letters*, 29(4), 593-597.
- Li, X., Ning, Y., Liu, D., Yan, A., Wang, Z., Wang, S., ... & Jia, Y. (2015). Metabolic mechanism of phenyllactic acid naturally occurring in Chinese pickles. *Food chemistry*, *186*, 265-270.
- Londero, A., Quinta, R., Abraham, A. G., Sereno, R., De Antoni, G., & Garrote, G. L. (2011). Inhibitory activity of cheese whey fermented with kefir grains. *Journal of Food Protection*, 74(1), 94-100.
- Martins, S., Mussatto, S. I., Martínez-Avila, G., Montañez-Saenz, J., Aguilar, C. N., & Teixeira, J. A. (2011). Bioactive phenolic compounds: production and extraction by solid-state fermentation. A review. *Biotechnology advances*, *29*(3), 365-373.
- Mu, W., Yu, S., Zhu, L., Jiang, B., & Zhang, T. (2012). Production of 3-phenyllactic acid and 4-hydroxyphenyllactic acid by Pediococcus acidilactici DSM 20284 fermentation. *European Food Research and Technology*, *235*(3), 581-585.
- Nascimento, G. G., Locatelli, J., Freitas, P. C., & Silva, G. L. (2000). Antibacterial activity of plant extracts and phytochemicals on antibiotic-resistant bacteria. *Brazilian journal of microbiology*, 31(4), 247-256.

- O'connell, J. E., & Fox, P. F. (2001). Significance and applications of phenolic compounds in the production and quality of milk and dairy products: a review. *International Dairy Journal*, *11*(3), 103-120.
- Rodríguez, H., Curiel, J. A., Landete, J. M., de las Rivas, B., de Felipe, F. L., Gómez-Cordovés, C., & Muñoz, R. (2009). Food phenolics and lactic acid bacteria. *International journal of food microbiology*, 132(2-3), 79-90.
- Rodríguez, N., Salgado, J. M., Cortés, S., & Domínguez, J. M. (2012). Antimicrobial activity of D-3-phenyllactic acid produced by fed-batch process against Salmonella enterica. *Food Control*, *25*(1), 274-284.
- Ryan, L. A., Zannini, E., Dal Bello, F., Pawlowska, A., Koehler, P., & Arendt, E. K. (2011). Lactobacillus amylovorus DSM 19280 as a novel food-grade antifungal agent for bakery products. *International journal of food microbiology*, *146*(3), 276-283.
- Sánchez-Maldonado, A. F., Schieber, A., & Gänzle, M. G. (2011). Structure–function relationships of the antibacterial activity of phenolic acids and their metabolism by lactic acid bacteria. *Journal of applied microbiology*, 111(5), 1176-1184.
- Schnürer, J., & Magnusson, J. (2005). Antifungal lactic acid bacteria as biopreservatives. *Trends in Food Science & Technology*, 16(1-3), 70-78.
- Soro-Yao, A. A., Brou, K., Amani, G., Thonart, P., & Djè, K. M. (2014). The use of lactic acid bacteria starter cultures during the processing of fermented cereal-based foods in West Africa: A review. *Tropical life sciences research*, 25(2), 81.
- Ström, K., Sjögren, J., Broberg, A., & Schnürer, J. (2002). Lactobacillus plantarum MiLAB 393 produces the antifungal cyclic dipeptides cyclo (L-Phe-L-Pro) and cyclo (L-Phe-trans-4-OH-L-Pro) and 3-phenyllactic acid. *Applied and environmental* microbiology, 68(9), 4322-4327.
- Swain, M. R., & Ray, R. C. (2016). Nutritional Values and Bioactive Compounds in Lactic Acid Fermented Vegetables and Fruits. *Lactic acid fermentation of fruits and vegetables.*
- Taylor, M. (Ed.). (2014). Handbook of natural antimicrobials for food safety and quality. *Elsevier.*
- Tremonte, P., Pannella, G., Succi, M., Tipaldi, L., Sturchio, M., Coppola, R., & Sorrentino, E. (2017). Antimicrobial activity of Lactobacillus plantarum strains isolated from different environments: a preliminary study. *International Food Research Journal*, 24(2).

- Tulini, F. L., Hymery, N., Haertlé, T., Le Blay, G., & De Martinis, E. C. (2016). Screening for antimicrobial and proteolytic activities of lactic acid bacteria isolated from cow, buffalo and goat milk and cheeses marketed in the southeast region of Brazil. *Journal of Dairy Research*, 83(1), 115-124.
- Valerio, F., Lavermicocca, P., Pascale, M., & Visconti, A. (2004). Production of phenyllactic acid by lactic acid bacteria: an approach to the selection of strains contributing to food quality and preservation. *FEMS microbiology letters*, *233*(2), 289-295.
- Valerio, F., Lavermicocca, P., Pascale, M., & Visconti, A. (2004). Production of phenyllactic acid by lactic acid bacteria: an approach to the selection of strains contributing to food quality and preservation. *FEMS microbiology letters*, *233*(2), 289-295.
- Varsha, K. K., Priya, S., Devendra, L., & Nampoothiri, K. M. (2014). Control of spoilage fungi by protective lactic acid bacteria displaying probiotic properties. *Applied biochemistry and biotechnology*, 172(7), 3402-3413.
- Vermeulen, N., Gánzle, M. G., & Vogel, R. F. (2006). Influence of peptide supply and cosubstrates on phenylalanine metabolism of Lactobacillus sanfranciscensis DSM20451T and Lactobacillus plantarum TMW1. 468. *Journal of agricultural and food chemistry*, 54(11), 3832-3839.
- Yoo, J. A., Lim, Y. M., & Yoon, M. H. (2016). Production and antifungal effect of 3-phenyllactic acid (PLA) by lactic acid bacteria. *Journal of Applied Biological Chemistry*, *59*(3), 173-178.

SPECIFIC OBJECTIVE 3

Evaluation of inhibition fungal growth in pita bread, by inserting fermented whey with lactic acid bacteria in its preparation.

Materials and methods

Chemicals and reagents

Wheat flour, sugar, salt, instant yeast and extravirgin olive oil were purchased in a shop market. Deionized water (<18 MX cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA. Buffered peptone water, potato dextrose agar (PDA), potato dextrose broth (PDB), De Man Rogosa-Sharpe medium (MRS Broth), were provided by Oxoid (Madrid, Spain).

Microorganisms and culture conditions

For bread contamination, the mycotoxigenic strains of *P. expansum CECT 2278* and *P. brevicopactum CECT 2316* purchased from the Colección Espanola de Cultivos Tipo (CECT) were used. All strains were maintained in PDA at room temperature (25 °C) until use.

Preparation of pita bread dough, inoculation and packaging

The experiment was conducted in the following steps:

1) Preparation of pita bread;

2) Contamination of the surface of bread with *P. expansum* and *P. brevicompactum*;

3) Packaging;

4) Observation of fungal growth.

1) *Preparation of pita bread*

Pita bread was prepared by adapting the method reported by Saladino *et al.* 2016. Commercial wheat flour 250 g, was mixed with 2.5 g of sugar, 20 mL extra virgin olive oil, 5 g of salt, 15 g of instant yeast and the quantity of water required to reach the recommended graphic consistency necessary to obtain a good quality of pita bread (125 mL). The dough was mixed for 12 minutes in the Simon extensometer mixer and the obtained dough was left rising for 30 min at 30°C in dark conditions. The formed dough was divided into 40 g portions and unrolled to the desired thickness of approximately 6 to 7 mm, and 4 cm in diameter. In total, the 9 dough of pita bread for each test were placed on a perforated greased plate and baked at 180 °C for 7 minutes in a muffle furnace (Memmert ULE 500 AO BARLOWORLD scientific S.A. , Madrid, Spagna). After baking, pita bread samples were cooled up to reach room temperature (20-22 °C, 1 h approximately) and then immediately prepared to be inoculated with fungi. The different steps of bread preparation are shown in the Figure 8.

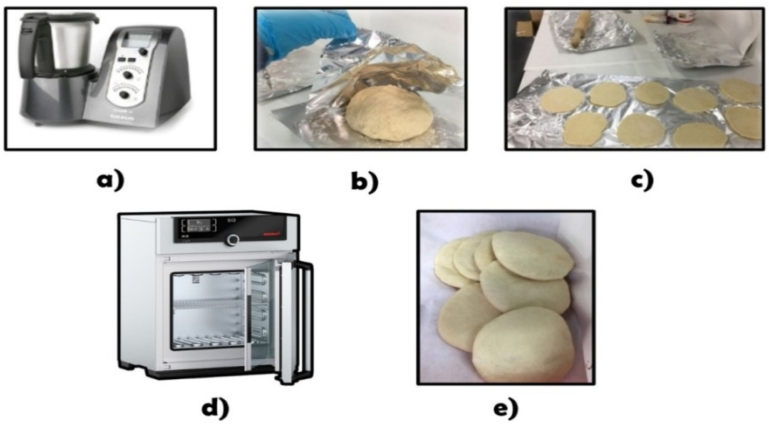


Figure 8. In the picture are shown the different steps of breads preparation.

Eight different typologies of bread were prepared:

- 27 replicas ofbread without synthesis additives: *positive control*;
- 27 replicas ofbread with calcium propionate 0.2%: *negative control*;
- 27 replicas ofbread with a concentration of 1% freeze-dried whey fermented by *L. plantarum* 220: ***test 1***;
- 27 replicas ofbread with a concentration of 1% freeze-dried whey fermented by *L. plantarum* 221: ***test 2***;
- 27 replicas ofbread with a concentration of 1% freeze-dried whey fermented by *L. plantarum* 223: ***test 3***;
- 27 replicas of bread with the replacement of 100% of water used in bread preparation with whey fermented by *L. planatrum* 220: ***test 4***;
- 27 replicas of bread with the replacement of 100% of water used in bread preparation with whey fermented by *L. planatrum* 221: ***test 5***;
- 27 replicas of bread with the replacement of 100% of water used in bread preparation with whey fermented by *L. planatrum* 223: ***test 6***;

2) *Artificial contamination with Penicillium expansum and Penicillium brevicopactum and natural contamination of bread*

The antifungal activity of fermented whey has been tested both in pita bread artificially contaminated with the chosen fungal spores that on pita bread exposed at natural contamination condition. The impact of antifungal inhibition of fermented whey on bread was evaluated following the method used by Ryan *et al.* 2011 with some modifications. Table 19 summarized all the experiments performed.

Table 19. In the table are shown the different experiments performed for the artificial and natural contamination of bread.

Experiments performed	Mycotoxigenic fungi used for inoculation of bread		
	P. expansum CECT 2278	*P. brevicopactum* CECT 2316	Without fungi: Natural contamination
Pita bread without synthesis additives			
Pita bread with calcium propionate 0.2%:			
Pita bread with a concentration of 1% freeze-dried whey fermented by *L. plantarum* 220			
Pita bread with a concentration of 1% freeze-dried whey fermented by *L. plantarum* 221			
Pita bread with a concentration of 1% freeze-dried whey fermented by *L. plantarum* 223			
Pita bread with the replacement of 100% of water used in bread preparation with whey fermented by *L. planatrum 220*			
Pita bread with the replacement of 100% of water used in bread preparation with whey fermented by *L. planatrum 221*			
Pita bread with the replacement of 100% of water used in bread preparation with whey fermented by *L. planatrum 223*			

After baking, 9 replicas for each typologies of pita bread were inoculated in 9 spots with 100 µl of a suspension containing $1x10^7$conidia/mL of the chosen fungi and 9 replicas for each typologies have followed a natural contamination (Figure 9). Conidial concentration was measured by optical density at 600 nm and adjusted to 10^7conidia/mL in PDB as reported (Kelly *et al.* 2006). For the evaluation of inhibition of fungal growth in bread, *Penicillium expansum* CECT 2278 and *Penicillium brevicopactum* CECT 2316 were used because are the most common species. *Penicillium* species were previously cultured in PDB liquid medium following the manufacturer's

specifications (27 g of product per 1 L of distilled water) and sterilization of the Selecta autoclave medium (Barcelona, Spain) at 121 ° C for 20 min.

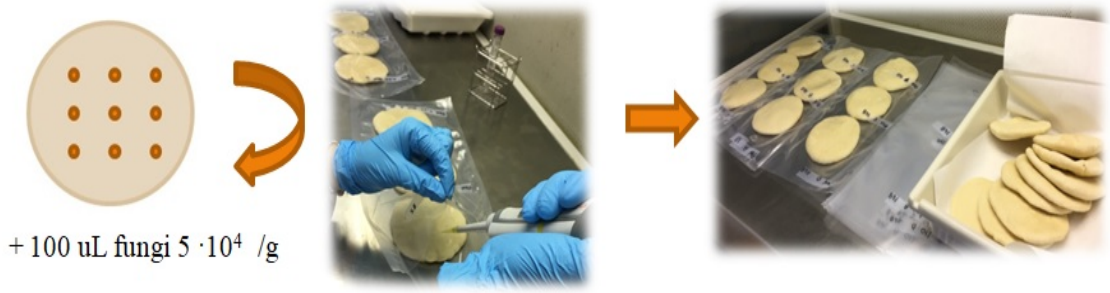


Figure 9. Inoculation of bread with *Penicillium* fungi.

3) *Packaging*

Finally, they were packed in sealed low-density polyethene bag with the help of a Sammic TS-150 (Basarte, Spain). All plastic bag was closed hermetically and incubated at room temperature during 10-20 days.

4) *Observation of fungal growth*

During that time, a visual control of the surface of the inoculated bread was carried out daily, identifying at a glance the possible growth or not of fungus and to establish the effect of the treatment on the shelf-life.

Antimicrobial activity of bread: determination of the fungal load of bread treated with fermented whey

Once the period of preservation of the bread samples was exceeded, a microbiological study was carried out, in order to determine the fungal contamination present in one of the bread samples. After 10 or 20 days for the artificially or natural contaminated of pita bread respectively, 10 g of bread were homogenized together with 90 mL of peptone water, previously

autoclaved, in a Stomacher IUL (Barcelona, Spain) for 30 seconds. From that, 3 serial decimal dilutions were prepared in glass tubes with 9 mL of peptone water. After 100 µL were plated out of each tube in PDA culture medium plates. The plates were incubated at 26 ° C, and the number of viable colonies counted after 72 h of incubation (Figure 10).

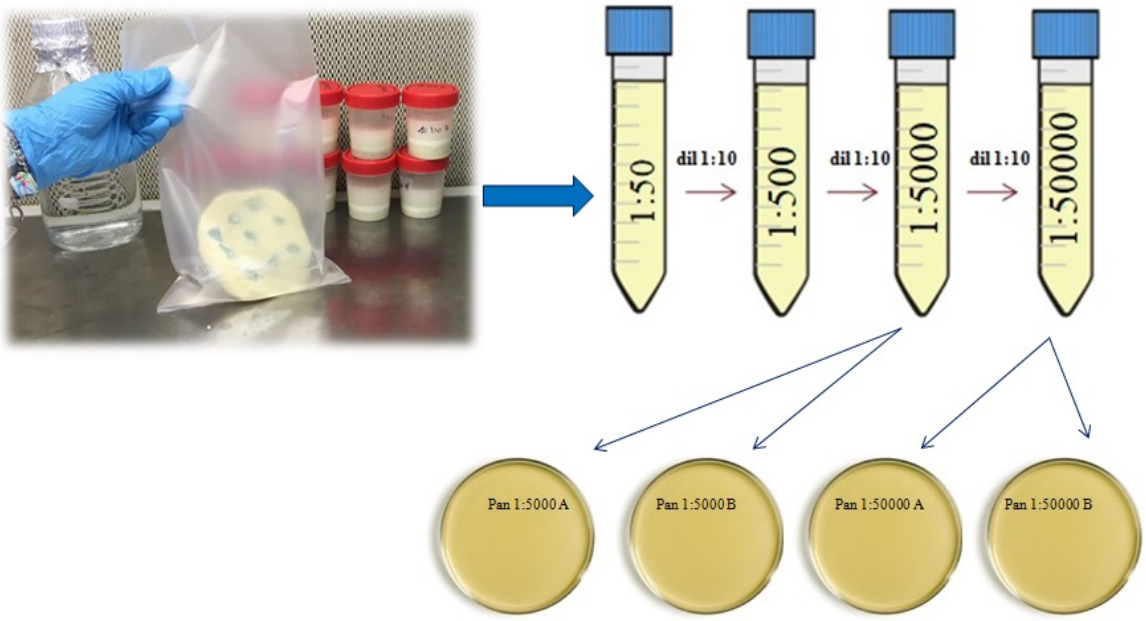


Figure 10. Antimicrobial activity of bread samples after the period of preservation.

Evaluation of the sensorial acceptability of bread produced with the fermentates

Sensorial evaluation of the different typologies of bread was performed by means of a nine-point Hedonic scale. The scale ranges from dislike (1) and extremely to like (9). The sensory evaluation of the bread was performed by 25 assessors to whom were presented coded samples in random order and were asked to evaluate the flavour of each sample in the order provided. The responses of the assessors to the flavour of the bread were then tallied, after which the averages and standard deviations were calculated.

Statistical analysis

Statistical analysis of data was performed using the Tukey's test. Results are expressed as mean values ± s.e. mean. The chosen significance level was $P < 0.01$.

Results and discussion

Artificial contamination with Penicillium expansum and brevicopactum

In recent years, the interest in food biopreservation has increased. Based on previous performed experiments, mycotoxigenic strains of *P. expansum* and *P. brevicompactum* were employed for studying the shelf-life improvement of bread. To simulate a real situation in which the products enter in contact only with the spores naturally present in the environment, an experiment without inoculation by fungi was performed. The results of the different experiments are shown in table 20.

In the positive control (bread prepared without additives), the visual growth of *P. expansum* and *P. brevicompactum* in the surface of pita bread started in the second day of the preservation period, whereas in the negative control (bread prepared with additives), the period of visual fungal growth is increased to sixth day. An important increment of the shelf-life was observed in the pita bread preparated adding whey fermented by *L. plantarum*. In this case, the visual fungal growth of *P. brevicompactum* in pita bread prepared adding 1% of freeze-dried fermented whey started on the fourth day of storage, whereas in pita bread prepared replacing water with sweet fermented whey, the fungal growth was observed between the ninth and tenth days, obtaining a total shelf life of eighth-nine days.

Similar results were observed also for the mycotoxigenic fungi *P. expansum* with the only difference that the fungal growth of pita bread prepared adding 1% of the freeze-dried whey, started at the third day. The experiments carried out using *L. plantarum* during the fermentation process of whey, evidenced an increment of the shelf life of pita bread, compared with the positive control, of 1-2 and 7-8 days for freeze-dried and liquid fermented whey, respectively.

As regards the results obtained from the natural contamination of bread, a visual fungal growth was obtained on the fifth day for positive control and on the sixth day for negative control. An appreciable increase in the shelf life was obtained from pita bread prepared with the fermented whey. In the case of freeze dried whey, a visual fungal grown was reached at the seventh and eighth day, whereas in pita bread prepared replacing water with liquid sweet whey, a slight fungal growth was observed at nineteenth and twentieth day.Visual representation of pita bread inoculated with *P. brevicompactum* and *P. expansum* after 10 days of the period of preservation and pita bread subject to natural contamination at 20th day are shown in figure 11,12.

The results obtained in this work are according to contents reported by Gerez *et al.* 2009, in which the ability of lactic acid bacteria (LAB) to inhibit *Aspergillus*, *Fusarium*, and *Penicillium* was evaluated in bread preparation. Using LAB in bread preparation, an increase in the shelf-life of bread of 5 days compared to bread without LAB was observed. In addition, LAB in bread preparation cause a 50% reduction in the concentration of calcium propionate, a synthetic preservative. These results are also in agreement with a recent study carried out by Le Lay *et al.* 2016. In the latter work, different LAB led to delayed fungal growth after incorporation in milk bread rolls

preparation. Also Dal Bello *et al* .2007 have screened *Lactobacillus plantarum* for in vitro antimicrobial activity, and it was resulted to be active against spoilage moulds and bacteria. This LAB were tested for the ability to produce the antifungal compounds during sourdough fermentation and to produce bread of good quality and increased shelf-life.

Fungal spoilage is the main cause of substantial economic losses in packaged bakery products and might also be cosidered a source of mycotoxins, involving public health problems. In this context, LAB may be considered as an alternative for bio-conservation.

Table 20. Shelf-life of pita bread contaminated with *P. expansum*, *P. brevicompactum* and natural contamination.

Pita Bread Sample *P. expansum*	**Shelf-Life (days)**									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Positive control	-	+	+	+	+	+	+	+	+	+
Negative control	-	-	-	-	-	+	+	+	+	+
Test 1	-	-	-	+	+	+	+	+	+	+
Test 2	-	-	-	+	+	+	+	+	+	+
Test 3	-	-	-	+	+	+	+	+	+	+
Test 4	-	-	-	-	-	-	-	-	-	+
Test 5	-	-	-	-	-	-	-	-	+	+
Test 6	-	-	-	-	-	-	-	-	+	+

Pita Bread Sample *P. brevicompactum*	**Shelf-Life (days)**									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Positive control	-	+	+	+	+	+	+	+	+	+
Negative control	-	-	-	-	-	+	+	+	+	+
Test 1	-	-	+	+	+	+	+	+	+	+
Test 2	-	-	+	+	+	+	+	+	+	+
Test 3	-	-	+	+	+	+	+	+	+	+
Test 4	-	-	-	-	-	-	-	-	-	+
Test 5	-	-	-	-	-	-	-	-	+	+
Test 6	-	-	-	-	-	-	-	-	-	+

Pita Bread Sample without fungi	Shelf-Life (days)															
	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Positive control	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Negative control	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Test 1	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Test 2	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Test 3	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Test 4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Test 5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Test 6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+

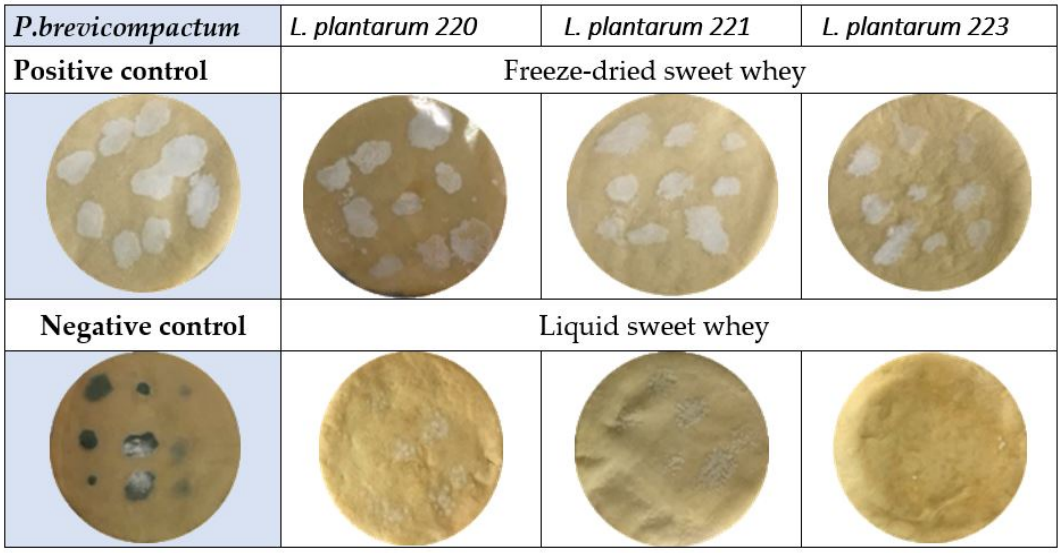


Figure 11. Visual representation of pita bread inoculated with *P.brevicompactum* and *P.expansum* after 10 days of the period of preservation.

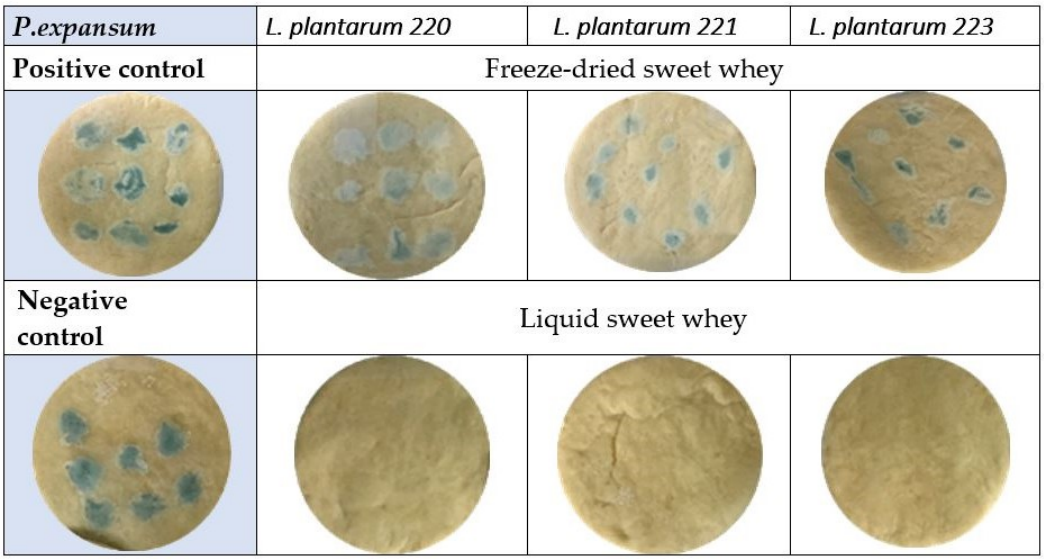


Figure 11. Visual representation of pita bread inoculated with *P.brevicompactum* and *P.expansum* after 10 days of the period of preservation.

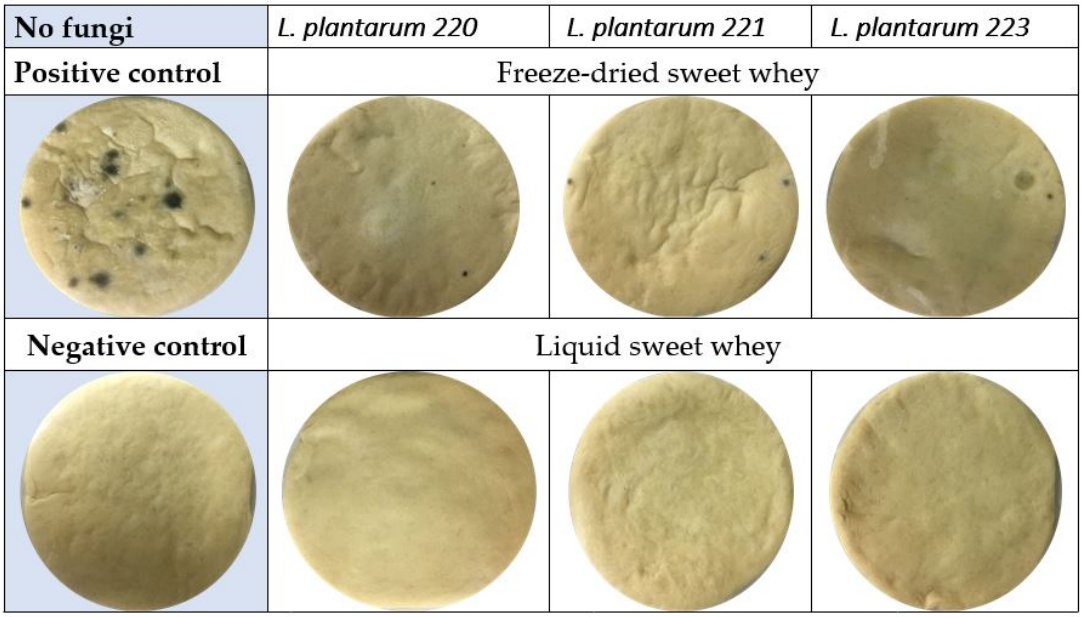


Figure 12. Visual representation of pita bread without inoculation with fungi after 20 days of the period of preservation.

Antimicrobial activity of bread: determination of the fungal load of bread treated with fermented whey

Exceeded the period of preservation of bread, a microbiological study was performed to determine the fungal contamination present in each one of the bread samples. Firstly, the value of pH of bread after homogenization in the stomacher was measured and the average with relative deviation standard are shown in table 21. A drop in pH was observed in the pita bread with the addition of fermented whey in the dough. A significative percentage of pH reductionwas observed in pita bread prepared totally replacing water with fermented whey of 100% of water used in bread preparation with fermented whey. The percentage of decrease was ranged between 17-25%, 18-28% and 13-20% for the pita bread contaminated with *P. expansum, P. brevicompactum* and natural contamination, respectively.

The antifungal effect would be related to both the nature of the organic acid produced during fermentation, such as lactic, acetic and PLA, or/and the low pH reached after fermentation. The decrease of pH is caused by releasing of weak organic acids such as propionic acid, which are mainly dependent on the undissociated molecule, whose fraction increases with a decrease in pH. The dependence of the antifungal activity of weak organic acids on pH has also been reported in several studies (Smith *et al.* 2004).

Table 21. Pita Bread: pH misured after preservation period.

Pita Bread: pH misured after preservation period	*P.expansum*	*P.brevicompactum*	Natural contamination
Positive control	5.79±0.072	6.08±0.03	6.11±0.02
Negative control	5.82±0.130	5.94±0.02	5.71±0.22
Test 1	5.36±0.015	5.59±0.01	5.51±0.14
Test 2	5.57±0.066	5.78±0.10	5.64±0.28
Test 3	5.42±0.010	5.55±0.05	5.54±0.06
Test 4	4.82±0.056	4.86±0.01	4.95±0.02
Test 5	4.94±0.038	5.03±0.04	5.08±0.01
Test 6	4.63±0.026	4.64±0.02	4.76±0.03

As regards the microbiological study of bread, a considerable percentage of reduction in fungal growth has been observed as shown in table 22. Adding 1% of freeze-dried fermented whey in bread preparation, a percentage of reduction ranged between 12-23% was obtained, whereas with the replacement of 100% of water used in bread preparation with whey fermented by LAB the percentage of reduction has a highest peak of increase ranged from 42 to 92% for the different typologies of test performed.

Table 22. Antimicrobial activity and % reduction of fungal growth

Pita Bread Sample	Fungi					
	P. *expansum*		P. *brevicopactum*		Without fungi	
	log ufc/g	% reduction	log ufc/g	% reduction	log ufc/g	% reduction
Positive control	4.52±0.44^{a}	-	3.30±0.07^{ab}	-	3.77±0.09^{a}	-
Negative control	4.37±0.15^{ab}	3 ± 3	2.78±0.44^{a}	-	3.11±0.13^{ab}	17 ± 3
Test 1	4.00±0.16^{ab}	12 ± 4	2.34±0.42^{ab}	16 ± 15	2.91±0.05^{bc}	23 ± 4
Test 2	4.00±0.27^{ab}	11 ± 6	2.33±0.27^{ab}	16 ± 10	3.00±0.12^{ab}	20 ± 6
Test 3	3.67±0.25^{ab}	19 ± 6	2.15±0.12^{b}	23 ± 4	2.96±0.13^{b}	21 ± 6
Test 4	0.57±0.90^{c}	87 ± 20	0.39±0.43^{c}	86 ± 16	1.20±0.90^{d}	68 ± 20
Test 5	0.31±0.57^{c}	93 ± 13	0.86±1.01^{c}	69 ± 36	2.17±0.50^{c}	42 ± 13
Test 6	0.73±0.66^{c}	84 ± 15	0.23±0.36^{c}	92 ± 13	0.56±0.53^{d}	85 ± 14

Sensory quality

Sensory evaluation of the bread was performed by 25 assessors for the different typologies of prepared samples. No significant difference (p>0.05) was observed regarding the texture of the different typologies of analyzed bread. As regards the flavours, this study showed that the differences between pita bread elaborated with various types of fermented whey and control bread could be perceived by an untrained panel. In fact, the positive control has demonstrated the highest average score for flavour (7.1, like slightly), followed by negative control (6.6, neither dislike no like), test 1, 2, and 3 (6.5, neither dislike nor like e like slightly), and finally the test 4, 5 and 6 (6.3, neither dislike no like). According to the one-way ANOVA, significant differences ($p > 0.05$) occurred between the tallied responses of the flavour of the bread.

References:

- Dal Bello, F., Clarke, C. I., Ryan, L. A. M., Ulmer, H., Schober, T. J., Ström, K., ... & Arendt, E. K. (2007). Improvement of the quality and shelf life of wheat bread by fermentation with the antifungal strain Lactobacillus plantarum FST 1.7. *Journal of Cereal Science*, *45*(3), 309-318.
- European Union (EU). (2011). Commission regulation (EU) No 1129/2011 of 11 november 2011 amending annex II to regulation (EC) No 1333/2005 of the European parliament and of the council by establishing a union list of food additives. *Official Journal of the European Union*, L295, 92
- Gerez, C. L., Torino, M. I., Rollán, G., & De Valdez, G. F. (2009). Prevention of bread mould spoilage by using lactic acid bacteria with antifungal properties. *Food control*, *20*(2), 144-148.
- Kelly, S., Grimm, L. H., Bendig, C., Hempel, D. C., & Krull, R. (2006). Effects of fluid dynamic induced shear stress on fungal growth and morphology. *Process Biochemistry*, *41*(10), 2113-2117.
- Le Lay, C., Mounier, J., Vasseur, V., Weill, A., Le Blay, G., Barbier, G., & Coton, E. (2016). In vitro and in situ screening of lactic acid bacteria and propionibacteria antifungal activities against bakery product spoilage molds. *Food Control*, *60*, 247-255.
- Saladino, F., Luz, C., Manyes, L., Fernández-Franzón, M., & Meca, G. (2016). In vitro antifungal activity of lactic acid bacteria against mycotoxigenic fungi and their application in loaf bread shelf life improvement. *Food Control*, *67*, 273-277.
- Smith, J. P., Daifas, D. P., El-Khoury, W., Koukoutsis, J., & El-Khoury, A. (2004). Shelf life and safety concerns of bakery products—a review. *Critical reviews in food science and nutrition*, *44*(1), 19-55.

SPECIFIC OBJECTIVE 4

Evaluation of biological activities including ACE-inhibitory, antioxidants, and Fe-binding in order to use this by-product for the formulation of a nutraceutical.

Materials and methods

Chemicals and reagents

The compounds 2,2-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), N-Hippuryl-His-Leu substrate, angiotensin-converting enzyme, hippuric acid, ferrozine, ferrous chloride, glutathione, were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Methanol, ethanol, trifluoroacetic acid, ethyl acetate, formic acid and acetonitrile. HPLC grade was purchased from Merck (Darmstadt, Germany). All other chemicals and reagents were of analytical grade. Deionized water (<18 MX cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Centricon Amicon with a cut-off of 3 kDa was provided by Microtech Srl (Buccinasco, MI).

Purification sample

An aliquot of 250 mg of freeze-dried fermented whey was solubilized in 1 mL of 0.1% TFA aqueous solution. The sample was purified on SPE C18 columns, previously activated, and then eluted with 3 mL of a mixture of acetonitrile/0.1% formic acid in water (70:30, v/v). The eluate was further purified on Centricon Amicon with a cut-off of 3 kDa to obtain a permeate consisting of small bioactive compounds as reported in the literature to show greater bioactivity. After purification, the permeate was lyophilized and finally re-suspended in 500 μL of water for subsequent analysis

(Aschaffenburg 1959). The purified and diluted samples were subjected to the biological test for the evaluation of antioxidant activity (ABTS), antihypertensive activity (ACE inhibitory) and iron binding activity.

ABTS radical cation scavenging assay

The method reported by Xiao *et al* .2014 was used to analyze the ABTS radical cation ($ABTS^{\bullet+}$) scavenging activity. $ABTS^{\bullet+}$ was obtained by the reaction of a 7 mM aqueous solution of ABTS with 2.45 mM aqueous solution of potassium persulphate which was performed in dark conditions at room temperature for 16 h prior to use. The $ABTS^{\bullet+}$ solution was diluted with ethanol to arrive at an absorbance value of 0.70 (±0.02) at 734 nm. $ABTS^{\bullet+}$ solution (1 mL, with an absorbance of 0.700 ± 0.050) was added to the tested samples (0.1 mL, opportunely diluted) and mixed thoroughly. The reactive mixture was allowed to stand at room temperature for 2.5 min and the absorbance was immediately recorded at 734 nm. Ethanolic solutions of known trolox concentrations were used for calibration. The results were expressed as millimoles of trolox equivalents TE per kg of dry weight.

ACE inhibitory activity

Evaluation of ACE inhibitory activity was assayed using the method described by Wu & Ding *et al.* 2002 with slight modifications. A sample solution of 25 µL (500 mg/mL) was mixed with 50 µL of 5 mMN-Hippuryl-His-Leu substrate HHL solubilized in 0.1 M borate buffer (pH 8.3) containing 0.3 M NaCl; the mixture was incubated at 37 °C for 10 min. After that, 20 µL of ACE solution (100 mU/mL) was added. The mixture was then re-incubated for 60 min at 37 °C. The reaction was stopped by adding 100 µL of 1 M HCl, and the mixture was centrifuged at 14 000 rpm (Thermo Fisher CL21R, Italy) for 10 minutes. The amount of hippuric acid (HA) in the

supernatant was determined by reversed-phase HPLC (Jasco MD-4016, Italy) using a Kinetex C18 column (5 μm, 4.6 × 250 mm, Phenomenex, USA) and detected using an UV-VIS detector at 228 nm. The mobile phase consisted of two solvents: solvent A was water with 0.1% trifluoroacetic acid and solvent B was acetonitrile with 0.1% trifluoroacetic acid. A linear gradient was used as follows: 0–8 min, 20%–50% B; 8–12 min, 50%–70% B; 12–14 min, 70%–20% B; 14–20 min, 20% B. The injection volume was 20 μL and the column temperature was 30 °C. The flow rate of the mobile phase was 1 mL/min. The percentage of ACE inhibition was calculated according to the following equation:

$$\%\,\text{ACE inhibition activity} = \left[\frac{\text{HA}_{\text{control}} - \text{HA}_{\text{sample}}}{\text{HA}_{\text{control}}}\right] \times 100$$

where $\text{HA}_{\text{control}}$ and $\text{HA}_{\text{sample}}$ are the peak areas corresponding to HA for the blank and for an inhibitor sample, respectively.

Ferrous ion chelating ability

The ferrous (Fe^{2+}) chelating ability was measured colorimetrically as described by Joshi *et al.* 2013 The solution of ferrous chloride 0.4 M (0.1 mL) was added to 1 mL (20 mg/mL) of freeze-dried fermented whey solubilized in distilled water followed by the addition of 0.2 mL of ferrozine 5 mM to initiate the reaction. The mixture was vortexed and the tubes were kept at room temperature for 10 min. The absorbance values were measured at 562 nm. Glutathione 1 mg/mL was used as positivecontrol. The ferrous ion chelating ability was calculated as follows:

$$\%\ \text{chelating ability} = \left[\frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}}\right] \times 100$$

where $\text{Abs}_{\text{control}}$ and $\text{Abs}_{\text{sample}}$ are the absorbance at 562 nm for the blank and for the sample, respectively.

Results and discussion

ABTS radical cation scavenging assay

Scientific research highlights the antioxidant capacity of peptides derived from food proteins. These antioxidant compoundsderived from proteins can offer a number of potential benefits and are also considered safer alternatives to synthetic antioxidants. Some of these peptides possess multiple biological activities, which can intensify their physiological efficacy and further reduce the disease risk (Meisel *et al.* 2004). Moreover, peptides can act in synergy with non-peptide antioxidants improving their protective effect. The recovery of bioactive peptides obtained from whey could be useful for production of specific functional or nutraceutical foods (Kitts & Weiler, 2003). In the present study, ABTS assay was used to measure the antioxidant activity on fraction whey obtained after purification through a 3 kDa cut-off membrane. Many authors have reported a large number of studies regarding the occurrence of antioxidant compounds in the fractions <3 kDa having less than 20 amino-acids. For this reason, particular interest was applied to these fractions having shown greater bioactivity. The results expressed as millimoles of trolox equivalents TE per kg of dry weight (mmol trolox equivalent per kg of d.w.) are shown in Figure 13. Considering the ABTS assay, whey fermented by the strains of *L. plantarum* 220 and *L. plantarum* 221 resulted in notably significant increases, during the 72 h of treatment. Whey fermented by *L. plantarum* 220 showed anincrease in the antioxidant activity

of 34% from 24 to 72 h of treatment and an increase of 27% compared to the control, while whey fermented by *L. plantarum* 221 showed an increase of 37% from 24 to 72 h of treatment and an increase of 15% compared to control. Similar results regarding the increase of antioxidant capacity during fermentation were reported in the literature by other authors (Hafeez *et al.* 2014). On the other hand, whey fermented with the strain of *L. plantarum* 748 showed the highest activity after 24 h of fermentation exhibiting at this fermentation time an increase in the antioxidant activity of 20% compared to those of the sample at 72 h of fermentation. Comparing the antioxidant activity of *L. plantarum* 748 with no fermented whey an increase of 46% was observed at the same fermentation time (24 h). However, it has been reported that *L. plantarum* strains are able to excrete different endopeptidases in fermentation media. The activities of various endopeptidases in medium containing proteins may result in the generation of different antioxidant compounds (Liu *et al.* 2011).

In recent studies conducted by Moreno-Montoro *et al.* 2017 and Aguilar-Toalá *et al.* 2017 the antioxidant activity of milk fermented by *L. plantarum* was analyzed. Their results highlighted, according to the evidence reported in the literature, that the highest antioxidant capacity measured by ORAC was found in the fraction of fermented milk derived from the cation exchange membrane permeate with molecular weight <3 kDa. In another study conducted by Osuntoki *et al* .2010 the antioxidant activity of fermented whey by *Lactobacillus* isolated from Nigerian food products was evaluated and the highest level of antioxidant activity was observed in the whey fermented with the strain of *L. brevis* isolated from wara, a dairy product. This is probably due to the strain being better adapted to the milk substrate, in contrast to the other no dairy isolated. In this experiment, the measurement of radical scavenging activity was conducted using the DPPH

test. The antioxidant activity monitored from 4 to 24 h of fermentation showed an increase in the antioxidant capacity of whey during fermentation. Several studies related to the production of antioxidant compounds in products fermented by LAB indicated that the development of radical scavenging activity was a strain-specific characteristic and that radical scavengers were related to proteolysis (Virtanen *et al.* 2007, Hernández-Ledesma *et al.* 2005, Gupta *et al.* 2009).

Though numerous studies have reported antioxidant activity in *Lactobacillus*, a direct comparison of results is difficult because of the variety of methods used, the various ways in which the results are expressed, the use of non-standardized inoculum size and various other differences.

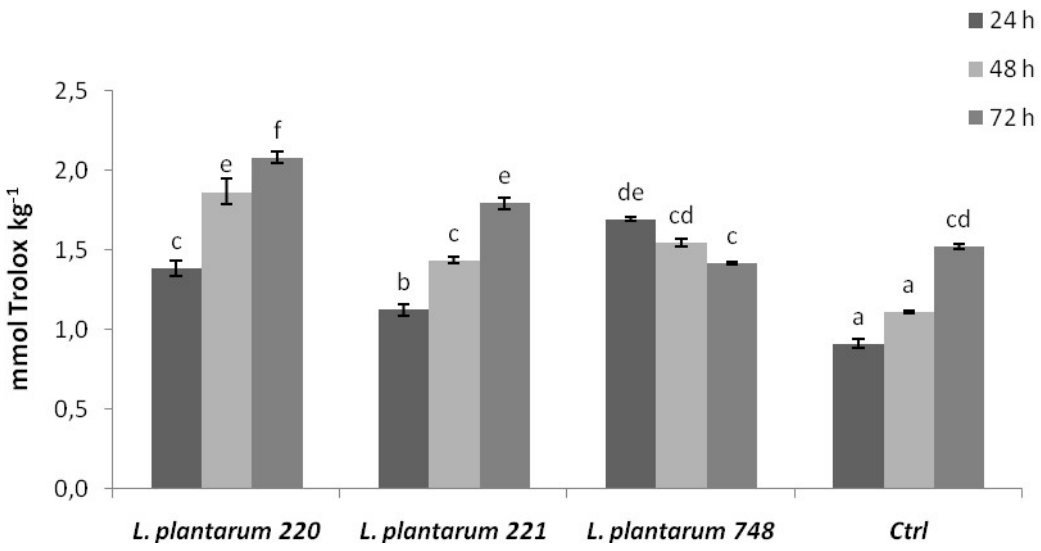


Figure 13. The radical scavenging activity of <3KDa whey fraction obtained during fermentation by LAB. In the figure, whey fermented with three strains of *L. plantarum* and control sample at triple fermentation (24, 48, 72h).

ACE inhibitory assay

Angiotensin-converting-enzyme (ACE) is a central component of the renin–angiotensin system (RAS), which has an importantfunction in cardiovascular

homeostasis. In RAS, the rennin has the capability to cut the liver-derived precursor, angiotensinogen, into a decapeptide: angiotensin I. The enzyme ACE catalyzes cleavage of angiotensin I to angiotensin II (a vasoconstrictor factor). Furthermore, ACE inactivate the vasodilative peptides bradykinin and kallidin, and stimulates the release of aldosterone, which increases blood pressure (Campbell, 2003).

In this study, ACE inhibitory activity was measured on whey fermented by *L. plantarum* 220, *L. plantarum* 221 and *L. plantarum* 748 at the respective fermentation times (24, 48, 72 h). The results shown in Figure 14 were expressed as the inhibition percentage compared to the control. ACE inhibition activity obtained by whey fermented with different strains of *L. plantarum* ranged between 67% and 85%. For all samples, ACE inhibitory activity increased proportionally to the fermentationtime (from 24 to 72 h), evidencing the highest activity at 72 h by the strain of *L. plantarum* 220.

In the literature, Gonzalez-Gonzalez *et al.* 2011 reported that the ACE-inhibitory activity of milk fermented with probiotic strains increased with fermentation reaching the highest value (85%) after 48 h of incubation. In particular, *L. plantarum* NCIMB 8826 inoculated in milk produces an increase in antihypertensive activity from 24 to 48 h. In contrast, Pihlanto *et al.* 2010 observed that *L. acidophilus* ATCC 4356 and *L. jensenii* ATCC 25258 strains, inoculated in milk, showed a high ACE inhibitory activity after 24 h of fermentation demonstrating that some strains are capable of producing high bioactivity at 24 h of fermentation. For this reason, our experimental protocol has provided a fermentation time of up to 72 h in order to monitor the trend of this biological activity. The results obtained for the ACE inhibitory activity of fermented whey samples were in accordance with the reported literature.

In fact, Pereira *et al.* 2017 reported the ACE-inhibitory effect of a drink prepared with goat's whey fermented by *L. casei*. The drink showed no significant differences in terms of acidity compared to the control, but showed instead a significant increase in the ACE inhibitory activity, due to proteolysis of the whey proteins over 24 h of fermentation. In particular, an ACE inhibitory activity almost comparable to captopril (ACE inhibitory drug) was observed after 24 h of fermentation time. Also, Pihlanto *et al.* 2010 studied fermented milk to test in vitro ACE inhibition of 25 different LABs at different times of fermentation. The measure of ACE inhibitory activities varied from 5 to 74%, and 8 out of 25 strains did not produce measurable ACE inhibition also modifying fermentation parameters such as pH. This result confirms that ACE inhibition could be closely connected to bacterial growth and proteolysis. Therefore, it is evident that, during fermentation, especially with strains of *L. plantarum*, compounds having potential biological activities, including the ACE inhibitory activity, were generated (Hernandez-Ledesma *et al.* 2005).

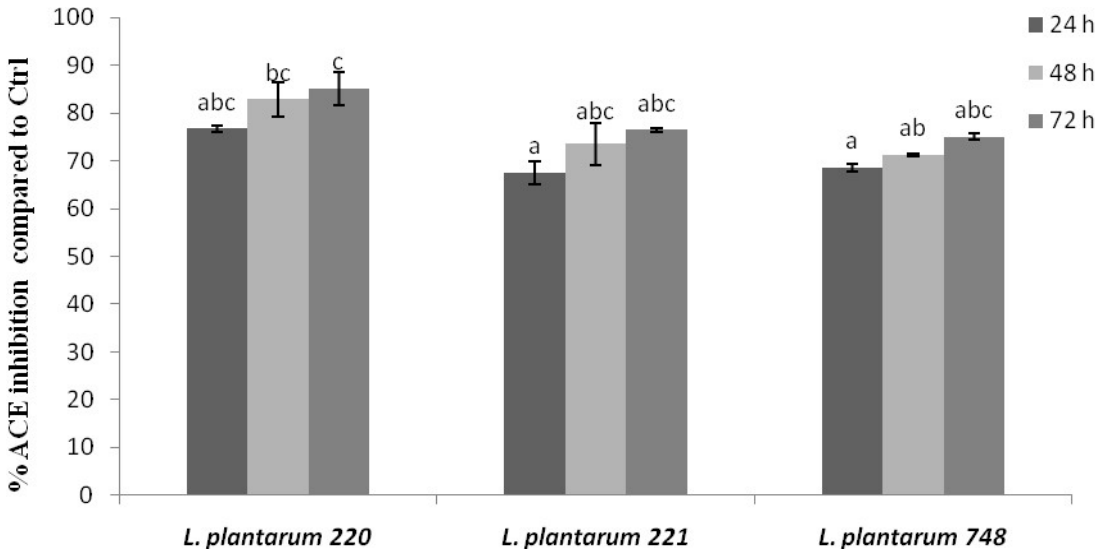


Figure 14. ACE inhibitory activity obtained from <3KDa whey fermented by three strains of *L. plantarum*at 24, 48 and 72h. The results were expressed in percentage of inhibition compared to control.

Ferrous ion chelating ability

It has been recognized that transition metal ions are involved in many oxidation reactions in vivo. Ferrous ions (Fe^{2+}) can catalyze Haber–Weiss reaction and induce superoxide anion to form more hazardous hydroxyl radicals. Hydroxyl radicals react rapidly with the near biomolecules and cause severe damage. It has been observed that the scavenging of hydroxyl radicals by antioxidant compounds was efficacious mainly via the chelating of metal ions (Pignatello *et al.* 2006). In the herein reported data, the metal chelating ability was determined by measuring the reduction rate of red color, which was formed by ferrozine with ferrous ions. The iron chelating activity was expressed as a percentage compared to the positive control represented by glutathione (1 mg/mL). Our results are shown in Figure 15, in the whey fermented by *L. plantarum* 220 and *L. plantarum* 221 the iron-chelating activity increased linearly with the fermentation time, reaching a maximum value after 72 h of fermentation. Fermentation induces the structural breakdown of proteins, leading to the liberation or synthesis of various compounds responsible also for iron chelating activity (Mokoonlall *et al.* 2016). This piece of evidence is discussed in a report prepared by Hur *et al.* 2014 regarding the effect of fermentation in plant-based foods and the correlation of this activity with the fermentation process. Even though investigations on whey have been conducted widely by several authors, there is limited information about the iron binding capability of this matrix after fermentation with microorganisms. Among the studies in the literature, the evidence of major iron binding activity correlated to compounds with lower molecular weight was reported. More particularly, O'Loughlin *et al.* 2015 investigated the iron chelating activity of two fractions of WPI at different degrees of hydrolysis (DH). They reported a 1 kDa permeate at 10%

DH and a 30 kDa retentate at 5% DH. These results showed that the capability of chelating iron was 84.4 μM EDTA and 8.7 μM EDTA for 1 kDa and 30 kDa fractions, respectively. Thus, it was suggested, according to our results, that the lower molecular weight of the WPI fractions was associated with higher iron chelating capability.

Regarding our results, whey fermented by *L. plantarum* 748 showed an inverse trend, generating the highest iron chelating activity after 24 h of fermentation (55%) and a significant decrease after 72 h (18%). Similar results were reported by Abubakr *et al.* 2012 who evaluated the iron binding activity of whey after fermentation by LAB. They showed the highest iron binding capacity after 24 h of fermentation, with a decrease from 97.6 to 41.8%in the following 48 hours, because of a high number of phosphoseryl serine groups which have greater affinity for iron. An interesting result was also obtained by Silva *et al.* 2015 who studied the iron chelation activity of whey protein hydrolyzedenzimatically with alcalase, pancreatin or flavourzyme, showing an increase of iron solubility at pH 7 following the interaction between aminoacid residues of casein derived phosphopeptides with metal irons. The main advantages of using LAB against enzymatic solution involves the decrease of cost and disposal problem keeping food safety.

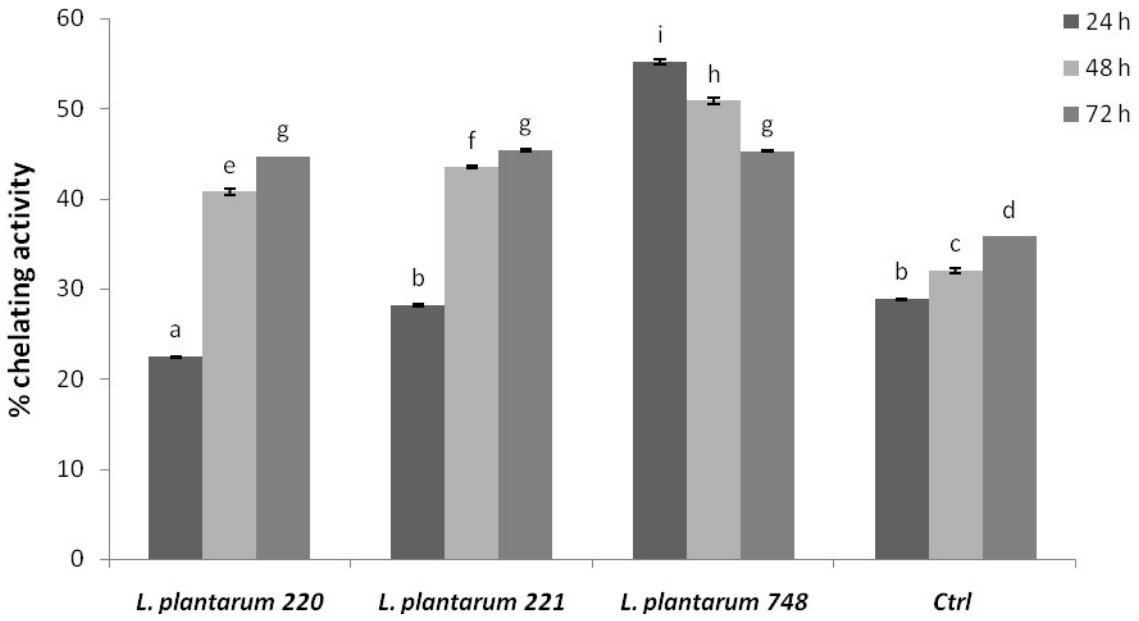


Figure 15. Iron chelating activity percentage of different samples of <3KDa whey fraction fermented by three strains of *L. plantarum* (220, 221, 748) at three different times of fermentation 24, 48 and 72h is illustrated in the figure.

References:

- Abubakr, M. A., Hassan, Z., & Imdakim, M. M. A. (2012). Antioxidant activity of lactic acid bacteria (LAB) fermented skim milk as determined by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and ferrous chelating activity (FCA). *African journal of microbiology research*, 6(34), 6358-6364.
- Aguilar-Toalá, J. E., Santiago-López, L., Peres, C. M., Peres, C., Garcia, H. S., Vallejo-Cordoba, B., & Hernández-Mendoza, A. (2017). Assessment of multifunctional activity of bioactive peptides derived from fermented milk by specific Lactobacillus plantarum strains. *Journal of dairy science*, 100(1), 65-75.
- Aschaffenburg, R., & Drewry, J. (1959). New procedure for the routine determination of the various non-casein proteins of milk. *XV Intern. Dairy congr*, 3, 1631-1637.
- Caetano-Silva, M. E., Bertoldo-Pacheco, M. T., Paes-Leme, A. F., & Netto, F. M. (2015). Iron-binding peptides from whey protein hydrolysates: Evaluation, isolation and sequencing by LC–MS/MS. *Food research international*, 71, 132-139.
- D. J. Campbell, Int. J. Biochem. Biotechnol., 2003, 35, 784–791.
- De Souza Pereira, Á. M., de Farias, D. R. B., de Queiroz, B. B., de Caldas Nobre, M. S., Cavalcanti, M. T., Salles, H. O., ... & Buriti, F. C. A. (2017). Influence of a Co-culture of Streptococcus thermophilus and Lactobacillus casei on the Proteolysis and ACE-Inhibitory Activity of a Beverage Based on Reconstituted Goat Whey Powder. *Probiotics and antimicrobial proteins*, 1-10.
- Gonzalez-Gonzalez, C. R., Tuohy, K. M., & Jauregi, P. (2011). Production of angiotensin-I-converting enzyme (ACE) inhibitory activity in milk fermented with probiotic strains: Effects of calcium, pH and peptides on the ACE-inhibitory activity. *International dairy journal*, 21(9), 615-622.
- Gupta, A., Mann, B., Kumar, R., & Sangwan, R. B. (2009). Antioxidant activity of Cheddar cheeses at different stages of ripening. *International Journal of Dairy Technology*, 62(3), 339-347.
- Hafeez, Z., Cakir-Kiefer, C., Roux, E., Perrin, C., Miclo, L., & Dary-Mourot, A. (2014). Strategies of producing bioactive peptides from milk proteins to functionalize fermented milk products. *Food Research International*, 63, 71-80.

- Hernández-Ledesma, B., Miralles, B., Amigo, L., Ramos, M., & Recio, I. (2005). Identification of antioxidant and ACE-inhibitory peptides in fermented milk. *Journal of the Science of Food and Agriculture*, 85(6), 1041-1048.
- Hur, S. J., Lee, S. Y., Kim, Y. C., Choi, I., & Kim, G. B. (2014). Effect of fermentation on the antioxidant activity in plant-based foods. *Food chemistry*, 160, 346-356.
- Joshi, R., Sood, S., Dogra, P., Mahendru, M., Kumar, D., Bhangalia, S., & Saxena, A. K. (2013). In vitro cytotoxicity, antimicrobial, and metal-chelating activity of triterpene saponins from tea seed grown in Kangra valley, India. *Medicinal Chemistry Research*, 22(8), 4030-4038.
- Kitts, D. D., & Weiler, K. (2003). Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. *Current pharmaceutical design*, 9(16), 1309-1323.
- Liu, C. F., Tseng, K. C., Chiang, S. S., Lee, B. H., Hsu, W. H., & Pan, T. M. (2011). Immunomodulatory and antioxidant potential of Lactobacillus exopolysaccharides. *Journal of the Science of Food and Agriculture*, 91(12), 2284-2291.
- Meisel, H. (2004). Multifunctional peptides encrypted in milk proteins. *Biofactors*, 21(1-4), 55-61.
- Mokoonlall, A., Nöbel, S., & Hinrichs, J. (2016). Post-processing of fermented milk to stirred products: Reviewing the effects on gel structure. *Trends in Food Science & Technology*, 54, 26-36.
- Moreno-Montoro, M., Olalla-Herrera, M., Rufián-Henares, J. Á., Martínez, R. G., Miralles, B., Bergillos, T., & Jauregi, P. (2017). Antioxidant, ACE-inhibitory and antimicrobial activity of fermented goat milk: Activity and physicochemical property relationship of the peptide components. *Food & function*, 8(8), 2783-2791.
- O'Loughlin, I. B., Kelly, P. M., Murray, B. A., FitzGerald, R. J., & Brodkorb, A. (2015). Molecular characterization of whey protein hydrolysate fractions with ferrous chelating and enhanced iron solubility capabilities. *Journal of agricultural and food chemistry*, 63(10), 2708-2714.
- Osuntoki, A., & Korie, I. (2010). Antioxidant activity of whey from milk fermented with Lactobacillus species isolated from Nigerian fermented foods. *Food Technology and Biotechnology*, 48(4), 505-511.

- Pignatello, J. J., Oliveros, E., & MacKay, A. (2006). Advanced oxidation processes for organic contaminant destruction based on the Fenton reaction and related chemistry. *Critical reviews in environmental science and technology*, 36(1), 1-84.
- Pihlanto, A., Virtanen, T., & Korhonen, H. (2010). Angiotensin I converting enzyme (ACE) inhibitory activity and antihypertensive effect of fermented milk. *International Dairy Journal*, 20(1), 3-10.
- Virtanen, T., Pihlanto, A., Akkanen, S., & Korhonen, H. (2007). Development of antioxidant activity in milk whey during fermentation with lactic acid bacteria. *Journal of applied microbiology*, 102(1), 106-115.
- Wu, J., & Ding, X. (2002). Characterization of inhibition and stability of soy-protein-derived angiotensin I-converting enzyme inhibitory peptides. *Food research international*, 35(4), 367-375.
- Xiao, Y., Xing, G., Rui, X., Li, W., Chen, X., Jiang, M., & Dong, M. (2014). Enhancement of the antioxidant capacity of chickpeas by solid state fermentation with Cordyceps militaris SN-18. *Journal of Functional Foods*, 10, 210-222.

3.CONCLUSIONS

Whey deriving from the dairy industry represents a waste product of great impact. Whey fermented by some strains of lactic acid bacteria produce inhibition of fungal growth of species belonging to the *Penicillium*, *Aspergillus* and *Fusarium* genera both in liquid and solid assay. This study suggests the possibility to reuse the fermented whey in the food industry. In particular, freeze-dried whey fermented by *L. plantarum* 220 and sweet whey fermented by *L.plantarum* 220 and 223 gives the best results in terms of antifungal activity, suggesting its use as a natural antimicrobial compound to control fungal contaminants and extend the shelf-life of foods and/or feedstuffs. It is, therefore, desirable to continue to expand our understanding of the effectiveness of fermented whey by LAB in order to estimate their efficacy for future applications in food model systems.

In addition, whey compounds obtained during fermentation process using LABs, have shown remarkable bioactivities in term of antioxidant, ACE-inhibitory and iron-binding activity, particularly the best results were obtained using *L. plantarum* 220 in the fermentation process. Even though, in current literature exists information on the various bioactivities of food protein-derived peptides, there is limited information on in vivo health-promoting effects, bioavailability, pharmacokinetics, molecular mechanisms of action and possible use in human disease. So, compounds derived during the fermentation process of whey could have potential application in human disorder, although future research efforts should be directed toward evaluation of risks for consumers prior to commercialization of new nutraceutical.

Acknowledgements

Al termine di un percorso cosi importante mi sento in dovere di ringraziare una serie di persone che direttamente o indirettamente hanno reso speciale questo momento.

Un ringraziamento speciale, al Direttore del Dipartimento di Farmacia, il Prof. Novellino, alla coordinatrice del corso di Dottorato in Scienza del Farmaco, la Prof. Maria Valeria D'Auria per la sua precisione ed attenzione nei confronti degli studenti del corso.

Ringrazio infinitamene il mio tutor, il Prof. Alberto Ritieni, per la disponibilità, la gentilezza e la fiducia che ha avuto nei miei confronti nel corso di questi anni. Ringrazio Giulia ed Anna per avermi dato i giusti consigli per accrescere le mie conoscenze scientifiche. Ringrazio Luigi, collega ed amico, per essere stato sempre presente, per il supporto morale e per tutte le volte che ha ascoltato il mio strano modo di lamentarmi a loop senza mai lamentarsi di conseguenza. A tutto il gruppo L6, grazie per avermi dato l'opportunità di vivere quest'esperienza di crescita professionale e personale.

A Jordi, Julliermina, e tutto il gruppo di lavoro di Valencia, in particolare a Peppe, il mio supervisor, per avermi concesso l'opportunità di imparare, nonostante la microbiologia fosse per me un mondo nuovo, tantissime nuove cose. Anche con tremila piastre e provette da riempire in un solo giorno mi sono divertita un sacco. Ti stimo per la sua voglia di fare, sempre pronto a tirare fuori un progetto e metterlo in pratica il secondo dopo. Ringrazio Vittoria la mia collega italiana a Valencia, amica di disavventure e compagna delle serate in laboratorio.

A Yelko, grazie per avermi dato gli strumenti per affrontare tutto questo percorso. Spesso mi dicevi: “Luana devi essere autosufficiente!” e ricordo che ci rimanevo tanto male perché pensavo: “Lui questa cosa la potrebbe risolvere in un secondo mentre io ci passerò giorni, uffaaaa!! Ti sono grata perché nonostante l’amicizia che si era creata tra noi, non mi hai agevolata permettendomi cosi di imparare ad essere autosufficiente!! Credo che non potrò mai ringraziarti abbastanza per questo, ma sono sicura che i tuoi studenti, i tuoi tesisti ed i tuoi futuri dottorandi avranno un grande maestro, e te ne saranno grati come me in questo momento. Sei per me un grande amico ed una grande guida, il mio esempio di scienza, spero un giorno di diventare quasi brava quanto te.

Alla mia compagna di stanza, di avventure, di disavventure, di esami, di dottorato, non so bene dove collocarti.. ma sicuramente sei quella persona che auguro a tutti di incontrare durante il proprio percorso di vita. La persona con la quale sarei potuta essere più competitiva di qualsiasi altra, ed invece sin da quel primo concorso durante il quale potevamo essere chi presa e chi no, non abbiamo mai avuto competizione. Ed oggi, dopo 3 anni posso chiaramente dire che senza te questo percorso non sarebbe stato lo stesso. Ti voglio bene Carmina.

Ringrazio il gruppo di ricerca del 3 piano, ringrazio tutti per tutti i momenti condivisi: Roberto, Giuseppe, Fabrizia, Mari, Connie, Viviana e più di tutti il carissimo Prof. Tenore, persona splendida che ha sempre creduto in me sin da quando ero solo una tesista! …e tutti tutti tutti gli interni!!!!

Alle coinquiline, Maria José ed Irene spendide compagne di avventura che hanno reso fantastici i mesi trascorsi a Valencia ed alle 6 pazze coinquiline di

casa Astone, per aver assistito alla reclusione ecclesistica durante il periodo di tesi, mi siete state tanto vicino , grazie di tutto belle!!

Alle tesiste Chiara, Federica e Silvia per la dolcezza, la grinta, e il supporto sperimentale. Alle Croatine Ivana e Tena, con le quali ho condiviso poco tempo ma nonostante la distanza ed il tempo la loro presenza è una costante. Potranno veramente separarci Kilometri? Vi voglio bene.

Alle amiche di sempre, Marianna e Stefania, sempre presenti in ogni momento della mia vita. Grazie per essermi sempre accanto.

A Mimmo, Annamaria, Antonella, Guglielmo e la carissima Sig. Mariagrazia. Ai miei zii, e mia cugina Maria per la spensieratezza donatami quando stavo giù!

Un ringraziamento speciale alla mia FAMIGLIA: a mia mamma, la mia forza ed il mio punto debole, ti ringrazio per la pazienza, il supporto e quell'amore indissolubile che ci lega. A mio fratello Tommaso, a mia sorella Rosa e mio cognato Pasquale per la grande stima che hanno di me, e per essere stati sempre presente e disponibili anche ad ogni mia richiesta improvvisa. Alla mia piccola gioia, Francesca per la spensieratezza che mi ha dato tanta serenità in questi anni! Ed infine al mio boss, mio padre, che giorno dopo giorno mi ha dato quella forza per crederci sempre di più, quel supporto speciale che mi ha portata anche senza la sua presenza fisica a credere in me! Spero che oggi tu da lassù sarai fiero di me.

Grazie a tutti!

Luana