UNIVERSITÀ CATTOLICA DEL SACRO CUORE
MILANO

Dottorato di ricerca in Biotecnologie Molecolari
ciclo XXI
S.S.D: AGR16

ERYTHROMYCIN AND TETRACYCLINE RESISTANT LACTOBACILLI IN THE PRODUCTION OF A TYPICAL DRY SAUSAGE FROM THE NORTH OF ITALY

Tesi di Dottorato di: Daniela Zonenschain
Matricola: 3480164

Anno Accademico 2007/08

UNIVERSITÀ CATTOLICA DEL SACRO CUORE
MILANO

Dottorato di ricerca in Biotecnologie Molecolari
ciclo XXI
S.S.D: AGR16

ERYTHROMYCIN AND TETRACYCLINE RESISTANT LACTOBACILLI IN THE PRODUCTION OF A TYPICAL DRY SAUSAGE FROM THE NORTH OF ITALY

Coordinatore: Ch.mo Prof. Morelli Lorenzo

Tesi di Dottorato di: Daniela Zonenschain
Matricola: 3480164

Anno Accademico 2007/08

To my parents Leon and Edith, who represent for me an example of strength, perseverance, generosity and wisdom.
To my sisters Simone and Claudia, the best friends forever.
To my husband Fabio, the perfect partner for everything, showing me everyday the importance to leave my life in a positive way.
To my daughter Camilla, the best reason to wake up and smile every day.

Acknowledgements

This study has been carried out at the Institute of Microbiology from the Universià Cattolica del Sacro Cuore (Piacenza, Italy).

I am grateful to my supervisor Prof. Lorenzo Morelli for the scientific support and useful suggestions during the progress of this thesis and for having guided me in these years in many research projects.

Thanks to Dr. Annalisa Rebecchi for giving me good suggestions when writing this thesis and for the pleasant moments together in our project's meetings and job discussions.

Thanks also to my colleagues from the Microbiology lab and from the AAT (Advanced Analytical Technologies) company for the opportunity to share a process of development and for the friendship dedicated to me.

List of Contents

CHAPTER 1

GENERAL INTRODUCTION

1.1. The antibiotic resistance problem

About 50 years ago, antibiotics were introduced for the treatment of microbial diseases (Mathur and Singh, 2005). The widespread use of antibiotics has achieved a significant reduction in the morbidity and mortality associated with infectious diseases (Ammor et al., 2007). Their use has been extended to veterinary medicine, where they are employed as therapeutic agents and animal growth promoters (Levy and Marshall, 2004), and both provide a selective pressure on certain bacteria of animal origin, dependent on the spectrum of activity of the antimicrobial in question (Teale, 2002). Therapeutic usage of antimicrobials is important to prevent the epidemic spread of animal disease and to protect their welfare. It can also prevent the transfer of zoonotic disease from animals to man (Ungemach, 2000). The greatest threat to the use of antimicrobial agents for therapy of bacterial infections has been the development of antimicrobial resistance in pathogenic bacteria (Mathur and Singh, 2005) and the consequent increasing emergence of resistant bacteria in humans (Phillips et al., 2004).

The resistance gene reservoir hypothesis suggests that beneficial and commensal bacterial populations in food and the gastrointestinal tract of animals and humans may play a role in the transfer of antibiotic resistance (AR) (Salyers et al., 2004). To reduce the spread of such resistance, appropriate use of antimicrobials is important, as is the screening for AR in bacteria intended for use in food systems (European Commission, 2005).

AR has been shown to have occurred rarely in bacteria collected before the antibiotic era (Hughes and Datta, 1983). Shortly after the introduction of each new antimicrobial compound, emergence of antimicrobial resistance is observed (Levy, 1997). It is estimated that some

1–10 million tons of antibiotics have been released into the biosphere over the last 60 years (European Commission, 2005); this spread of AR genes throughout the human environment represents a major public health problem in developed and developing countries (Levy, 1997).

Antibiotic-resistant microorganisms are an increasing medical problem primarily attributed to the overuse of antibiotics. Indeed, a correlation between antibiotic use and resistance has repeatedly been reported (Normark and Normark, 2002; Turnidge, 2004). The magnitude of the problem is significantly increased by the possibility of bacteria to transfer resistance determinants horizontally and by the escalating increase in the use (overuse and misuse) of antibiotics, which has created an enormous selective pressure towards resistant bacteria (Levy, 1997).

The use of antibiotics in the food chain, mainly in food-producing animals, has contributed to the development and spread of resistant bacteria in the environment (Tenover and Hughes,1996). Thus, AR is a growing worldwide health-related problem, which has been recently defined as a shadow epidemic (Alliance for the Prudent Use of Antibiotics, http://www.apua.org).

The extensive use of antimicrobials has created also a selective pressure for point mutations and acquisition of mobile genetic elements encoding antimicrobial resistance leading to spread of a variety of antimicrobial resistance determinants (Teuber et al., 1999; Teuber, 2001).

Up to now, studies on the occurrence and spread of AR in bacteria and on the mechanisms involved in these resistances have focused on pathogenic microorganisms because they represent an immediate risk to public health (Rizzotti et al., 2005). Because non-pathogenic bacteria may also be a source for resistance genes that can spread to pathogens,

surveillance activities should include non-pathogenic as well as pathogenic bacteria (Aquilanti et al., 2007). In fact, growing interest has now been directed to the study of antibiotic-resistant commensal bacteria. Indeed, such microorganisms are often associated with animals and foods of animal origin, and they could endanger consumers as well. Moreover, AR genes are often located on mobile genetic elements, such as plasmids, transposons, and integrons, and this makes their intraspecific, interspecific, and intergeneric transfer possible (Sorum and L'Abée-Lund, 2002). Transfer of AR determinants in natural microenvironments between bacteria of diverse origins has been demonstrated by some authors (Cocconcelli et al., 2003; Kruse and Sorum, 1994). Therefore, food products containing commensal bacteria resistant to antibiotics can be considered potential vehicles for AR genes that can be spread to pathogens (Danielsen and Wind, 2003; Teuber and Perreten, 2000).

1.2. Antibiotic resistance mechanism

Antibiotics kill or inhibit susceptible bacteria leaving the resistant ones to proliferate. AR may be achieved by a number of different mechanisms, including (i) decreased uptake of the antibiotic, (ii) increased export of the antibiotic, (iii) inactivation or modification of the antibiotic target, (iv) introduction of a new antibiotic resistant target, (v) hydrolysis of the antibiotic, (vi) modification of the antibiotic, and (vii) prevention of activation of the antibiotic (Normark and Normark, 2002).

AR determinants may be vertically or horizontally spread in natural microbial communities. A vertical dissemination is mediated by the clonal spread of a particular resistant strain. For horizontal gene transfer in bacteria three mechanisms have been identified (Davison, 1999): the natural

transformation, involving the uptake and incorporation of free DNA from the extra cellular medium, conjugation, a cell contact dependent DNA transfer mechanism found to occur in most bacterial genera and transduction via bacteriophages. Resistances may be inherent to a bacterial genus or species (natural or intrinsic resistance) that results in an organism's ability to thrive in the presence of an antimicrobial agent due to an inherent characteristic of the organism. Intrinsic resistance is not horizontally transferable, and poses no risk in non-pathogenic bacteria (Mathur and Singh, 2005).

In contrast, acquired resistance is present in some strains within a species usually susceptible to the antibiotic under consideration, and might be horizontally spread among bacteria. Acquired resistance to antimicrobial agents can take place either from mutations in the bacterial genome or through the acquisition of additional genes coding for a resistance mechanism. These genetic changes alter the defensive functions of the bacteria by changing the target of the drug by changing the membrane permeability, by enzymatic inactivation of antibiotic, by active transport of antibiotics, by target modification (Davies, 1997), or by routing metabolic pathways around the disrupted point (Poole, 2002). Resistances are likely to have developed long before the clinical use of antibiotics. Such resistance genes may originate from the antimicrobial producers that carry resistance genes for protecting themselves from their antimicrobial products (Davies, 1997).

The transfer of resistance genes to pathogenic or opportunistic bacteria poses a serious threat, since infections caused by these microorganisms cannot be treated with common antibiotics (Normark and Normark, 2002; Phillips et al., 2004). Resistances are not virulence factors

by themselves, but infections with resistant microorganisms complicate the course of the diseases and put up the price of their treatment. They also duplicate average stays at hospitals and double morbidity and mortality (Levy and Marshall, 2004).

For several decades, studies of the selection and dissemination of ARs have mainly focused on clinically relevant bacterial species. More recently, the hypothesis has been advanced that commensal bacteria may act as reservoirs of antibiotic resistant genes found in human pathogens (Gevers et al., 2003b). Such reservoirs can be present in the intestines of farm animals exposed to antibiotics and may thus contaminate raw meat even when hygienic standards and regulations are complied with (Sorensen et al., 2001; Sundsfjord et al., 2001). The resistance gene reservoir hypothesis suggests that beneficial and commensal bacterial populations may play a role in the transfer of AR to pathogenic and opportunistic bacteria (Teuber et al., 1999; Salyers et al., 2004).

Non-pathogenic antibiotic-resistant bacteria like lactobacilli and enterococci are increasingly being isolated from poultry, swine, calf (Giraffa, 2002; Gevers et al., 2003a) and from healthy human faeces (Aarestrup et al., 2000). Bacteria involved in food fermentation may also constitute AR reservoirs (Giraffa, 2002; Danielsen and Wind, 2003; Franz et al., 2003). Raw meat and fermented foods are therefore potential vehicles for the spread of antibiotic resistant bacteria and/or AR along the food chain to the consumer raising major concerns with regard to food safety (Aarestrup et al., 2000; Donabedian et al., 2003; Franz et al., 2003).

Such reservoir organisms could be found in various foods and food products containing high densities of non-pathogenic bacteria as a result of their natural production process. In this way, the food chain can be

considered as an important route of transmission of antibiotic resistant bacteria between different environments as the animal and the human one. In this context, many countries are developing research programmes that aim at monitoring resistance in bacteria isolated from food animals (Tollefson et al., 1998; Mevius et al., 1999).

1.3. The lactic acid bacteria and antibiotic resistance

The lactic acid bacteria (LAB) are a group of microorganisms that can convert fermentable carbohydrates into lactic acid (Leroy and de Vuyst, 2004). Due to their facultative anaerobic nature, the members of this group are present in a wide range of environments. The most typical members are Gram-positive, aero tolerant catalase-negative organisms of the low C+G branch, belonging to the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Pediococcus* (Carr et al., 2002).

Many LAB species are involved in the manufacture and preservation of fermented feed and foods from raw agricultural materials (such as milk, meat, vegetables and cereals) in which they are present as contaminants or deliberately added as starters in order to control the fermentation process, having therefore a great economic importance. In addition, LAB contribute to the organoleptic and nutritional properties of fermented feed and foods (Leroy and de Vuyst, 2004), and ensure the stability of the products mainly by producing lactic acid, which prevents the growth of pathogens (Fontana et al., 2005, Morot-Bizot et al., 2006). Some LAB strains may also act as bio protective cultures by the production of antimicrobial compounds (bacteriocins), thus enhancing the safety of fermented sausages (Hugas et al., 1998). These bacteriocins are non toxic and meet the requirements for food preservatives (Al-Hamidi, 2004). Inoculation of the sausage batter with

a starter culture composed of selected LAB, improves the quality and safety of the final product and standardizes the production process (Hugas and Monfort, 1997).

LAB have a long history of safe use as food-processing aids and as probiotics (Salminen et al., 1998; Gevers, 2000; Egervärn et al., 2007b), which are now widely used to give consumers a health benefit (Bernardeau et al., 2007). The probiotic effects of lactobacilli in humans are well documented. Several recent reviews highlighted the benefits and limitations of their use in different medical and health-related areas: control of intestinal inflammation (Andoh and Fujiyama, 2006); alleviation of lactose intolerance (Levri et al., 2005), stimulation of the immune system (Cross, 2002), protection against urogenital infections (Merk et al., 2005), improvement of human health (Ljungh and Wadstrom, 2006); their value in treating infections during pregnancy (Lewis, 2006); their therapeutic role in gastroenterology (Young and Cash, 2006); management of allergic diseases (Boyle and Tang, 2006); control of antibiotic-related diarrhoea (McFarland, 2006) and prevention of urinary tract infections (Falagas et al., 2006).

Over the last decade, scientific understanding of lactobacilli (e.g. their metabolism and functions) has expanded considerably, opening the way to more reliable process control in production and an increasing range of industrial dairy applications as starters and adjunct starters/cultures (including probiotics) (Chamba and Irlinger, 2004), raising discussion of new safety aspects, one of them being the nature of acquiring and distribution of antimicrobial resistance genes (Cataloluk and Gogebakan, 2004).

Anyway, AR in LAB has gained increased attention during recent years (Danielsen and Wind, 2003; Delgado et al., 2005; Flórez et al., 2005;

Zhou et al., 2005) because of their broad environmental distribution associated with the fact that they may function as reservoirs of AR genes that can be transferred via the food chain or within the gastrointestinal tract to other bacteria, including human pathogens (Teuber et al., 1999; Gevers et al., 2000).

Food safety is a top priority for the European Communities, as indicated in the White Paper on Food Safety (Commission of European Communities, 2000), and it is regulated by Commission of European Communities directive 93/43/CEE (Council of the European Communities, 1993).

Because of their long-time use in various food and feed preparations, LAB have been given the so-called GRAS status (generally recognized as safe) (Salminen et al., 1998; Borriello et al., 2003). In practice, this means that such LAB strains are food-grade organisms without imposing a health risk for the consumers or the environment. However, there are several studies that have documented the presence and expression of virulence genes and/or AR genes in food-associated LAB (Salminen et al., 1998; Borriello et al., 2003; Teuber et al., 1999; Danielsen and Wind, 2003). Anyway, the potential health risk, due to the transfer of AR genes from LAB reservoir strains to bacteria in the resident microflora of the human gastrointestinal tract and hence to pathogenic bacteria, has not been fully addressed (Mathur and Singh, 2005). Therefore, it is very important to verify that probiotic and nutritional LAB strains consumed on a daily basis worldwide lack acquired antimicrobial resistance properties prior to considering them safe for human and animal consumption (Klare et al., 2007).

Lactobacilli are non spore-forming rods with a G-C content generally in the 33–55% range (Coenye and Vandamme, 2003). They are strictly fermentative, and have complex nutritional requirements (carbohydrates, amino acids, peptides, fatty acid esters, salts, nucleic acid derivatives, vitamins). Grown on glucose as a carbon source, lactobacilli may be homofermentative (producing more than 85% lactic acid) or heterofermentative (producing lactic acid, carbon dioxide, ethanol/or acetic acid in equimolar amounts) (Bernardeau et al., 2006). They are found in a variety of habitats such as the mucosal membranes of humans and animals (oral cavity, intestine and vagina), on plants and material of plant origin, (Bernardeau et al., 2007); they also constitute an important part of the natural microflora associated with fermented products (Gevers et al., 2003a).

Bacteria of the genus *Lactobacillus* are beneficial microorganisms of particular interest because of their long history of use (Holzapfel, 2002). Lactobacilli were among the first organisms used for processing foodstuffs (Konigs et al., 2000) and for preserving food by inhibiting invasion by other microorganisms that cause food borne illness or food spoilage (Adams, 1999); they play a crucial role in the production of fermented foods: vegetables, meats and particularly fermented dairy products (Bernardeau et al., 2007).

The use of selected species of lactobacilli as starter organisms in industrial food and feed fermentations has a long tradition (Bernardeau et al., 2006). Lactobacilli widely used in starter cultures or as probiotics in dairy products enter our intestines in large numbers and there interact with the intestinal microbiota. Because of their broad environmental distribution, these bacteria may function as vectors for the dissemination of antimicrobial

resistance determinants that via the food chain can be transferred to the consumer (Teuber et al., 1999). As a general rule, lactobacilli have a high natural resistance to bacitracin, cefoxitin, ciprofloxacin, fusidic acid, kanamycin, gentamicin, metronidazole, nitrofurantoin, norfloxacin, streptomycin, sulphadiazine, teicoplanin, trimethoprim/ sulphamethoxazole, and vancomycin (Danielsen and Wind, 2003).

Lactobacilli are generally susceptible to antibiotics inhibiting the synthesis of proteins, such as chloramphenicol, erythromycin, clindamycin and tetracycline, and more resistant to aminoglycosides (neomycin, kanamycin, streptomycin and gentamicin) (Charteris et al., 1998; Zhou et al., 2005). However, resistant strains to these agents have also been identified (Danielsen and Wind, 2003; Delgado et al., 2005; Flórez et al., 2005), and several genes providing such resistance have been studied; e.g., a chloramphenicol resistance *cat* gene has been found in *Lactobacillus reuteri* (Lin et al., 1996) and *Lactobacillus plantarum* (Ahn et al., 1992), different erythromycin-resistance genes (*erm*) (Cataloluk and Gogebakan, 2004; Aquilanti et al., 2007; Ammor et al., 2008), and a number of tetracycline resistance genes *tet* (K, M, O, Q, S, W) have been found in many species (Villedieu et al., 2003; Torres et al., 2005; Huys et al., 2006). *Lactobacillus* spp. isolated from fermented dry sausages have been reported able to harbour tetracycline resistance gene (*tet*(*M*)) (Gevers et al., 2003b) and transfer of macrolide resistance from *Lactobacillus* to enterococci in vivo has been documented by Jacobsen et al. (2007) indicating that *Lactobacillus* spp. may play a role in the spread of antimicrobial resistance.

Due to the multiplicity of methods available, there is a lack of agreement regarding the resistance–susceptibility breakpoints for most antibiotics in LAB. Antimicrobial susceptibility testing of LAB can be

performed by several methods, including agar disc diffusion and agar overlay disc diffusion, E-test, agar dilution and broth macro- and microdilution (Klare et al., 2005).The different methods used are an initial source of confusion since their results cannot be directly compared (Swenson et al., 1992). The culture medium can also influence the results of susceptibility assays (Huys et al., 2002; Matto et al., 2006). Variations in the cation content or the concentration of critical compounds such as thymine or folic acid can modify the results obtained; as can the inoculum size, the temperature, the incubation period, etc. In general, dilution methods and the E-test are preferred over diffusion tests providing inhibition zones, as the former techniques allow determination of MICs of antimicrobials that result in a more reliable indication of the intrinsic or acquired nature of a given resistance phenotype (Klare et al., 2007).

Many LAB require special growth conditions in terms of medium acidity and carbohydrate supplementation, and for this reason conventional media such as Mueller–Hinton and Iso-Sensitest (IST) agar or broth are often not suitable for susceptibility testing of non enterococcal LAB (Klare et al., 2007), and there is some concern about possible antagonistic interactions between MRS components and specific antimicrobial agents (Huys et al., 2002; Danielsen and Wind, 2003). Additionally, the low pH of MRS medium (pH 6.2 ± 0.2) could be responsible for decreased activities of some antibiotics, e.g., aminoglycosides (Klare et al., 2007). For this reason, Klare et al. (2005) developed a broth formula referred to as the LAB susceptibility test medium (LSM) for determining MICs of antibacterial agents of all major antibiotic classes for *Lactobacillus* species.

Phenotypic assays have now been complemented by molecular methods in which bacterial strains are directly screened for the presence of

AR determinants. These methods include amplification by PCR with specific primers for single or multiplex AR genes (Strommenger et al., 2003), real time PCR (Volkmann et al., 2004), or the use of DNA microarrays containing large collections of AR genes (Perreten et al., 2005).

1.4. Fermented sausages

Fermented sausages are the result of biochemical, microbiological, physical and sensorial changes occurring in a mixture of meat (Casaburi et al., 2007) and fat particles, salt, curing agents and spices, which have been stuffed into a casing, fermented (ripened) and dried (Fontana et al., 2005).

These changes can be summarized as follows: decrease in pH, changes in the initial microflora, reduction of nitrates to nitrites and the latter to nitric oxide, formation of nitrosomyoglobin, solubilisation and gelification of myofibrillar and sarcoplasmic proteins, proteolytic, lipolytic and oxidative phenomena, and dehydration (Casaburi et al., 2007).

There is a wide variety of dry fermented products on the European market as a consequence of variations in the raw materials, formulations and manufacturing processes, which come from the habits and customs of the different countries and regions (Talon et al., 2007). Slightly fermented sausages form a group of traditional Mediterranean products which have a pH of 5.3–6.2 and present a great regional diversity, both between and within countries (Aymerich et al., 2006; Talon et al., 2007).

In general, the qualitative characteristics of naturally fermented sausages are known to be largely dependent on the quality of the ingredients and raw materials, the specific conditions of the processing and ripening, and the composition of the microbial population (Aquilanti et al., 2007), the latter being influenced by the original microbial contamination of raw

materials, temperature, redox potential, pH and water activity of the fermentation process (Lucke, 1985). In this context, the knowledge and control of their typical in-house microflora and the production processes are critical in terms of their organoleptic characteristics and microbiological quality (Aymerich et al., 2003). Traditional dry sausages rely on natural contamination by environmental microflora. This contamination occurs during slaughtering and increases during manufacturing (Morot-Bizot et al., 2006; Talon et al., 2007).

LAB (*Lactobacillus* spp.) and CNS, represented by the *Staphylococcus* genera, are the dominant bacteria in the fermentation and ripening of sausages (Coppola et al., 2000, Aymerich et al., 2003.; Fontana et al., 2005; Rantsiou and Cocolin, 2006; Morot-Bizot et al., 2006) followed by moulds, enterococci and yeasts that are also important microorganisms involved in sausage fermentation (Casaburi et al., 2007; Villani et al., 2007). LAB are actively involved in the development of texture, colour, and flavour and exert a positive effect on the hygienic properties of the product, inhibiting pathogenic or spoilage flora by acidification or by production of antimicrobials (Aymerich et al., 1998).

It is well known that LAB, in particular lactobacilli, play an important role in meat preservation and fermentation processes (Fontana et al., 2005). Even when no starter cultures are used, LAB, which are usually present in low numbers ($10^2 \pm 10^3$ CFU/g) in raw meat, rapidly dominate the fermentation because of the anaerobic environment and the presence of NaCl, nitrate and nitrite and because of their ability to reduce pH by production of lactic acid from carbohydrates (Hammes and Knauf, 1994). Their ability to lower the pH and produce bacteriocins prevent the growth of pathogenic and spoilage microorganisms, improving the hygienic safety and

storage of meat products (Fontana et al., 2005), and also, they develop the desirable organoleptic properties of the final product (Parente et al., 2001), being responsible to the "tangy" flavour of sausages and to the production of large amounts of lactic acid and for the small amounts acetic acid (Molly et al., 1996).

The type of microflora that develops in sausage fermentation is often closely related to the ripening technique utilised. Sausage with a short ripening time has more lactobacilli from the early stages of fermentation, and an "acid" flavour predominates in the products, which are commonly sold after less than two weeks of ripening. The intensity of this flavour depends on the pH value, but, at a given pH, a high amount of acetic acid gives the product a less "pure" and more "sour" flavour (Montel et al., 1998). Longer ripening times and greater activity of microorganisms other than LAB, such as CNC and yeasts, lead to higher levels of volatile compounds with low sensory thresholds (Lucke, 1985).

Among LAB, *Lactobacillus sakei* and *Lactobacillus curvatus* are the species most frequently isolated from dry sausages (Cocolin et al., 2001; Parente et al., 2001; Torriani et al., 1990; Rantsiou et al., 2005), but also *Lactobacillus plantarum* is very often found (Aymerich et al., 2003; Coppola et al., 2000; Fontana et al., 2005; Rantsiou and Cocolin, 2006).

In Europe, fermented sausage manufacturing has a long tradition (Rantsiou et al., 2005). Even when the use of starter culture has become common in the manufacture of several types of fermented products, many typical fermented sausages are still produced with traditional technologies without selected starters (Fontana et al., 2005; Rantsiou et al., 2005; Casaburi et al., 2007). In this case, the required microorganisms originate from the meat itself or from the environment, and constitute a part of the so-

called ‘‘house-flora’’ (Santos et al., 1998). This is the case of Italy, where almost every region offers one or more of these products, some of which have been awarded Protected Designation of Origin (PDO) and Protected Geographical Indication labels (http://europa.eu.int /comm/agriculture/qual/en/pgi_03en.htm).

Foods that are typical of any region or area have their own peculiar characteristics that arise from the use of local ingredients and production techniques, which are deeply rooted in tradition and linked to the territory (Aquilanti et al., 2007); this is the case for the Piacentino salami.

The Piacenza territory (north of Italy) is characterized by a humid continental climate which does not present any excessive thermal variations, a natural environment particularly favorable for pork raising - for which green zones are required, with plenty of water protected from the direct sun beams and excessive heat – and, thus, ideal for the production of salami. The Piacentino salami is made of pork meat and fat only. These derive from pork born and raised at Emilia Romagna and Lombardy, while the zone of production comprises the entire Piacentino territory, where this product has been present for centuries.

The production process is held in four stages: first the greasy and thin parts are triturated together; then the material is mixed, to which is added salt, spices and wine in the perfectly adequate quantities; the mixed product obtained is then held in a natural casing, placed to dry in adequate places for about a week; finally, there is the stage of maturation, which is of about at least 45 days. The final product is presented in a cylinder form, weighting 400 grams to 1 Kg. The Piacentino salami must be placed for commercialization with the pertinent PDO seal, which attests to its origin and respect to the traditional production practices.

The aim of the study:

Saprophytic bacteria that acts as reservoirs of AR genes can be present in the intestines of farm animals exposed to antibiotics and may thus contaminate raw meat even when hygienic standards and regulations are complied with. Raw meat and fermented foods are therefore potential vehicles for the spread of antibiotic-resistant bacteria along the food chain to the consumer raising major concerns with regard to food safety. The aim of this study was to analyse the diffusion of AR in *Lactobacillus* isolated from a food chain of a fermented dry sausage and from the end products obtained from artisanal factories producing Piacentino salami.

CHAPTER 2

RESULTS

2.1. ERYTHROMYCIN AND TETRACYCLINE RESISTANT LACTOBACILLI IN THE PRODUCTION CHAIN OF AN ITALIAN SALAMI

This paper was submitted to the "*International Journal of Food Microbiology*" and is still subject to approval for publication

Erythromycin and tetracycline resistant lactobacilli in the production chain of an Italian salami

Running title: Erythromycin and tetracycline resistance in lactobacilli

Zonenschain Daniela[1,*], Rebecchi Annalisa[2], Callegari M. Luisa[2], Morelli Lorenzo[1,2]

[1] Istituto di Microbiologia, Facoltà di Agraria, Università Cattolica del Sacro Cuore, Via Emilia Parmense 84, 29100 Piacenza, Italy

[2] Centro Ricerche Biotecnologiche, Via Milano 24, 26100 Cremona, Italy

* Corresponding author: Istituto di Microbiologia, Facoltà di Agraria, U.C.S.C., Via Emilia Parmense 84, 29100, Piacenza, Italy. Tel.: +39 0523 599244; fax: +39 0523 599246.

E-mail address: daniela.zonenschain@unicatt.it (D. Zonenschain)

Abstract

The scope of this study was to assess the frequency of erythromycin and tetracycline resistant lactobacilli in the production chain of a Protected Designation of Origin dry sausage from the North of Italy by microbiological analyses of the skin, minced meat, and stools of eight swine, of the natural casing, and of the final product at days 0, 21, 35, and 45 of ripening. We isolated 426 colonies of lactobacilli from selective medium supplemented with erythromycin or tetracycline; these isolates were genetically ascribed to 92 different strains. *Lactobacillus plantarum* and *Lactobacillus sakei* were the most frequently species isolated from the process line while *Lactobacillus reuteri* was the predominant species in stools. Over 90% of process line strains were resistant to tetracycline and 59.1% to erythromycin. Double resistance was detected in 50% and 67.1% of strains from the process line and stools, respectively. The most frequent resistance genes in process line strains were *tet(M)* and *ermB* while *tet(W)* and *ermB* were common in strains isolated from stools. Thus, erythromycin and tetracycline resistant lactobacilli were widespread in the production chain and stools of swine; however, the number of these drug resistant bacteria in the end product was low.

Keywords: erythromycin and tetracycline resistance, *Lactobacillus*, production chain, fermented dry sausage

1. Introduction

Artisanal fermented sausages are traditional Mediterranean products that vary greatly within the different regions (Aymerich et al., 2006). Numerous studies have performed microbiological characterizations of traditional sausages produced in Greece, Italy, and Spain (Coppola et al., 1998; Samelis et al., 1998; Parente et al., 2001). In Italy, there are a great variety of natural fermented sausages and almost all are known only at the local or regional level (Casaburi et al., 2008).

In general, the qualitative characteristics of naturally fermented sausages are largely dependent on the quality of the ingredients and raw materials, the specific conditions of the processing and ripening, and the composition of the microbial population (Coppola et al., 1998; Parente et al., 2001). The latter is influenced by the original microbial contamination of raw materials, temperature, pH, and water activity during the fermentation process (Lucke, 1985), and by the season of production, considering that more species are detected in spring than in winter (Morot-Bizot et al., 2006). In this context, understanding and control of typical in-house microflora and production processes are critical in terms of the organoleptic characteristics of the sausage (Rantsiou and Cocolin, 2006) and its microbiological quality (Aymerich et al., 2006).

According to conventional and molecular microbiological studies, the ripening process of fermented sausages is dominated by lactic acid bacteria (LAB), represented mainly by *Lactobacillus sakei*, *Lactobacillus curvatus*, and coagulase-negative cocci represented by the *Staphylococcus* and *Kocuria* genera (Coppola et al., 2000; Cocolin et al., 2001; Fontana et al., 2005; Cocolin et al., 2006; Rantsiou and Cocolin,

2006), followed by enterococci, molds, and yeasts that are also important microorganisms involved in sausage fermentation (Villani et al., 2007).

Even when no starter cultures are used, LAB, which are usually present in raw meat in low numbers (10^2-10^3 CFU/g), rapidly dominate the fermentation because of the anaerobic environment, the presence of nitrate and nitrite, and because of their ability to reduce pH by the production of lactic acid from carbohydrates (Hammes and Knauf, 1994).

Antimicrobial agents have been used in animal feeds as growth promoters in Europe for nearly half a century and have contributed to the increasing emergence of resistant bacteria in humans (Phillips et al., 2004). Until now, studies on the occurrence and spread of antibiotic resistance (AR) in bacteria and on the mechanisms involved in this resistance have focused on pathogenic microorganisms because they represent an immediate risk to public health (Rizzotti et al., 2005).

Recently, it has been hypothesised that saprophytic bacteria present in the intestines of animals exposed to antibiotics might act as reservoirs of AR genes and that these organisms can contaminate raw meat even when hygienic standards and regulations are followed (Sorensen et al., 2001). The presence of AR genes in animals and food raises great concern because AR can be carried by mobile genetic elements such as plasmids, transposons, and chromosomal cassettes (Rowe-Magnus et al., 2001), and can occur by intra- and inter-specific and even inter-generic transfer (Gevers et al., 2003). The transfer of resistance genes to pathogenic or opportunistic bacteria renders them untreatable by common antibiotics (Phillips et al., 2004). Because bacteria involved in food fermentation might constitute reservoirs of AR genes (Danielsen and Wind, 2003), raw meat and fermented foods are potential vehicles

for the spread of AR to pathogens (Teuber and Perreten, 2000) and ultimately to the consumer (Sorensen et al., 2001), raising major concerns with regard to food safety (Donabedian et al., 2003).

The aim of this study was to identify, at the species and strain level, tetracycline and erythromycin resistant *Lactobacillus* colonies collected from swine stools and from the production chain of an Italian fermented sausage (Piacentino salami) and to evaluate the diffusion of some AR genes in these isolates.

2. Material and methods

2.1. Fermented sausages production and sampling procedures

The Piacentino salami is a fermented Italian dry sausage produced in the North of Italy (Piacenza province). It is manufactured according to the traditional technique, without the addition of starter cultures. The batter is stuffed into natural casings and ripened as follows: one week of fermentation under relative humidity (RH) ranging from 40-90% at 15-25°C and six weeks of drying at 70-90% RH and 12-19°C. For commercial sale, it must receive the pertinent Protected Designation of Origin (PDO) seal, which attests to its origin and traditional production practices.

The samples analysed in this study were withdrawn at various steps of the production chain from one factory producing Piacentino salami. All samples came from the same lot of eight pigs from which meat, stool, and skin specimens were collected. After slaughtering, swine were washed with water at 65°C; samples were collected from the skin of each swine before and after washing by swabbing a 100 cm^2 area. The natural

casing was also studied. The final product was analysed after 0, 21, 35, and 45 days of ripening.

2.2. Enumeration of lactobacilli

Ten grams of each matrix was homogenized in 90 ml of saline/peptone water (8g/L NaCl, 1 g/L bacteriological peptone, Oxoid) using a Stomacher apparatus (400 Circulator, PBI, Milan, Italy) at 260 rpm for 2 min. Samples of casing, minced meat and dry end product were analyzed in duplicate. For the 16 skin samples, the swabs were placed in 10 ml of saline/peptone water and vortexed for 10 s. Serial dilutions of the homogenates were prepared using the same diluents, and aliquots of 100 µl of these were inoculated onto de Man, Rogosa, Sharpe (MRS) agar (Oxoid) using the spreading method and incubated at 30°C in anaerobiosis for 48 h. Growth medium was supplemented with 4 µg/ml erythromycin (Sigma) or 8 µg/ml tetracycline (Sigma) to screen for AR (concentrations of antibiotics were based on the breakpoints values defined by European Food Safety Authority [EFSA, 2005]).

The colonies on each plate were counted and 3-30 (about 10%) colonies of lactobacilli were randomly selected, streaked on MRS agar plates, and subcultured in tubes containing MRS supplemented with the antimicrobial agent at the same concentrations used for the initial isolation. The antibiotic resistant isolates were purified and stored at -80°C in 25% glycerol.

161 *2.3. DNA extraction*

162 DNA of pure lactobacilli cultures was extracted using the Puregene
163 DNA Purification Kit (Gentra Systems, Minneapolis, USA) following
164 manufacturer's instructions.
165
166 *2.4. Species identification*
167 Amplified Ribosomal DNA Restriction Analysis (ARDRA),
168 described by Ventura et al. (2000), was performed to identify species of
169 *Lactobacillus*. In order to confirm species identification, PCR products
170 from one representative of each specie were purified using the Wizard
171 SV Gel and PCR Clean-Up system according to the package insert
172 (Promega Corporation, Madison, Wis., USA) and sequenced at the
173 Biomolecular Research (BMR) Centre, University of Padova, Italy. The
174 identities of these isolates were determined by comparison against
175 sequences in the GenBank DNA database
176 (http://www.ncbi.nlm.nih.gov/).
177
178 *2.5. Strain typing*
179 Repetitive Extragenic Palindronic (REP) PCR using the (GTG)5
180 primer was used to identify lactobacilli isolates at the strain level as
181 already been described by Gevers et al. (2001). The patterns obtained
182 were analysed using Gel Compare 4.0 software (Applied Math, Kortrijk,
183 Belgium).
184
185 *2.6. Determination of phenotypic antimicrobial resistance*
186 The phenotypic antimicrobial resistance of a strain to a certain
187 antibiotic was determined as the minimum inhibitory concentration

(MIC). MICs were determined by the broth microdilution method using the standardized LAB susceptibility test medium (LSM) broth formulation, which ensures adequate growth of the test organisms and is essentially consisted of a mixture of Iso-Sensitest broth medium (Oxoid) (90%) and MRS broth medium (10%) adjusted to pH 6.7 as previously described by Klare et al. (2005).

Tetracycline was tested at 4-512 µg/ml, and erythromycin was tested at 0.25-512 µg/ml. Bacteria were inoculated into LSM broth to a final concentration of 3×10^5 CFU/ml and incubated at 37°C for 48 h in anaerobiosis. The MIC was defined as the lowest antibiotic concentration that resulted in no visible growth.

MIC50 and MIC90 are defined as MICs inhibiting 50% and 90% of the isolates tested, respectively, and they were determined to the antimicrobials named above for the 92 strains tested in this study.

2.7. PCR detection of antimicrobial resistance genes

The presence of genes involved in resistance to tetracycline (*tet(L)*, *tet(M)*, *tet(S)*, *tet(W)*) and macrolide-lincosamide-streptogramins (*ermB*, *ermC*) was determined by PCR. About 10 ng of bacterial DNA was used for PCR in a total volume of 25 µl containing 0.5 µM of each primer and Megamix (Microzone Limited, UK). Positive control DNA was included in each PCR reaction, and a negative control reaction containing no template was included in each run. Primer pair sequences, target genes, amplicon sizes, positive control strains and PCR protocol references are listed in Table 1.

To confirm the results, PCR products of each AR gene found in this study were chosen at random, purified using the Wizard SV Gel and PCR

Clean-Up system according to the package insert (Promega Corporation, Madison, Wis., USA), and sequenced at the BMR Centre. The BlastN program was used for nucleotide sequence analysis.

3. Results

3.1. Enumeration of lactobacilli

We analysed samples of casing, minced meat, dry end product after 0, 21, 35, and 45 days of ripening, skin before and after washing, and swine stools in order to evaluate the presence of AR lactobacilli along the production chain of a dry sausage.

In the minced meat and at day 0, 10 cfu/g of lactobacilli were detected on tetracycline or erythromycin containing medium. After 21 days the counts increased to about 10^6 cfu/g. The counts on tetracycline plates remained stable until the end of ripening while the counts on erythromycin plates increased from one log. In the casing, only erythromycin resistant isolates (10^5 cfu/g) were detected. No colonies (<10 cfu/g) were present on the plates inoculated from the skin before or after washing in the presence of either antibiotic. About 10^8 cfu/g were present on both types of antibiotic-containing plates inoculated with stool samples. The colony counts for all samples grown on the selective medium supplemented with both antibiotics are shown in Figure 1.

3.2. Species identification

A total of 426 colonies of lactobacilli were isolated and nine different species were detected: *Lactobacillus reuteri, Lactobacillus plantarum, L. sakei, Lactobacillus paracasei, Lactobacillus amylovorus, Lactobacillus brevis, Lactobacillus fermentum, Lactobacillus johnsonii,* and *L.*

242 *curvatus*. The species most frequently found along the process line were
243 *L. sakei* and *L. plantarum* (Table 2).
244 The only species isolated along the entire process line was *L. reuteri*
245 and this was also the only species found in the minced meat. In the
246 casing, the predominant species were *L. sakei* and *L. plantarum* while at
247 day 0 only *L. plantarum* was present. From the 21st day through the end
248 of ripening *L. sakei, L. plantarum, L. reuteri,* and *L. paracasei* were
249 always present while *L. brevis* and *L. curvatus* were found only during
250 certain periods of ripening. At the end of ripening, *L. sakei* was the
251 predominant species, representing 55% of the isolates at this point.
252 In stools, *L. reuteri, L. plantarum, L. sakei, L. amylovorus, L. brevis,*
253 *L. fermentum*, and *L. johnsonii* were present; *L. reuteri* was most
254 prevalent (70%).
255
256 *3.3. Strain typing*
257 REP pattern analysis demonstrated the presence of 92 different
258 strains of *Lactobacillus*, 70 were found in stools and 22 at the different
259 points in the production chain. At the beginning of ripening only one
260 strain was found while 12 different strains were found in the final
261 product.
262 Four strains were found in the casing: *L. sakei* 73 (61.3%) and *L.*
263 *sakei* 109 (3.2%), *L. plantarum* 2 (32.3%), and *L. reuteri* 27 (3.2%). *L.*
264 *sakei* 73 survived throughout processing and was present in the end
265 product, albeit at reduced numbers. *L. plantarum* 2 was also detected at
266 day 0 (it was the only strain present at this point) and it was present until
267 the 35th day but the number of colonies had reduced one log; it was not
268 present in the end product. *L. reuteri* 27 was present in increasing

numbers until the 35th day, but in the end product the number of colonies had decreased, although not significantly.

3.4. Determination of phenotypic antimicrobial resistance

The complete distribution of MICs of the two antimicrobial agents tested for the 92 lactobacilli isolates is described in Table 3.

The MICs for tetracycline ranged between 16 and 512 μg/ml (57% of strains had an MIC of 512 μg/ml), while the erythromycin MICs ranged between 0.25 and 512 μg/ml. Considering the EFSA (2005) breakpoints values, strains were considered to be phenotypically resistant when the MIC of tetracycline reached 32 μg/ml for *L. plantarum*, or 8 μg/ml for the other lactobacilli, and the MIC of erythromycin reached 4 μg/ml. According to these criteria, 13 (59.1%) strains were phenotypically resistant to erythromycin, 20 (90.9%) to tetracycline, and 12 (50%) were resistant to both. Some of these AR strains were found at different points in the production chain. In stools, 48 (68.5%) and 69 (98.5%) strains were phenotypically resistant to erythromycin and tetracycline, respectively, and 47 (67.1%) were doubly resistant. The number of phenotypically resistant lactobacilli present at each point of the production chain and in stools are shown in Table 4.

All but three *L. plantarum* strains were resistant to tetracycline. Resistance to erythromycin was found in all strains of *L. johnsonii* and *L. curvatus*, in 83% of *L. brevis* and *L. fermentum*, and in 73% of *L. reuteri*. All erythromycin resistant strains were doubly resistant except for two *L. plantarum* strains. The distribution of phenotypic antimicrobial resistance among the strains is reported in Table 5.

296 *3.5. PCR detection of antimicrobial resistance genes*

297 The number of AR genes found at each point in the production chain
298 and in stools is shown in Table 4. The results of PCR identifying the AR
299 genes present in these lactobacilli are reported in Table 5.

300 In the 20 strains from the production chain that were phenotypically
301 resistant to tetracycline, *tet(M)* was the most common tetracycline-
302 resistance gene detected, harboured by 12 strains (60%), and was found
303 at almost all sampling points except in the minced meat. This was the
304 predominant *tet* gene found in *L. plantarum* and *L. paracasei*. Two
305 (10%) of the 20 strains carried *tet(W)*, and this gene was particularly
306 present at 35 and 45 days of ripening. The *tet(S)* gene was found in two
307 (10%) of the remaining tetracycline resistant strains, both belonging to
308 the *L. plantarum* species. Four (20%) strains did not carry any of the *tet*
309 genes analysed in this study and *tet(L)* was not detected in any of the
310 strains. Nine of the 13 strains (69%) from the production chain that were
311 phenotypically resistant to erythromycin carried the *ermB* gene. This
312 gene was found at almost all points in production except at day 0, and it
313 was particularly prevalent in *L. sakei* and *L. reuteri*. No strains held the
314 *ermC* gene and four (30.7%) strains did not carry any of the *erm* genes
315 considered in this study. Of the 12 doubly resistant strains, five held
316 *ermB* and *tet(M)*, two carried *ermB* and *tet(W),* and one carried *ermB* and
317 *tet(S)*. Two strains carried multiple *tet* genes.

318 Of the 69 tetracycline resistant strains found in stool samples, 46
319 (66.6%) held the *tet(W)* gene, 16 (23.1%) carried *tet(M)*, only one (1.4%)
320 harboured *tet(S)*, and 9 (13%) strains did not carry any of the *tet* genes
321 analysed in this study. Of the 48 erythromycin resistant strains, 44
322 (91.6%) carried the *ermB* gene, three (6.2%) harboured *ermC* gene, and

only one did not carry any of the *erm* genes analysed in this study. Finally, of the 47 doubly resistant strains, 29 held *ermB* and *tet(W)*, 11 carried *ermB* and *tet(M)*, and two carried *ermC* and *tet(W)*. Three strains carried multiple *tet* genes.

4. Discussion

The extensive use of antibiotics for treating microbial infections in humans, animals, and plants, and as growth promoters in animal feed has led to the spread of AR in commensal microorganisms, creating large reservoirs of AR genes in non-pathogenic bacteria that are linked to the food chain (Aquilanti et al., 2007). These genes have the potential to be transferred both horizontally and vertically; however, the implications of these findings with regard to public health remain unclear (Phillips et al., 2004). Nevertheless, the food chain has become recognized as one of the main routes for the transmission of AR between animal and human populations (Teuber et al., 1999).

Most investigations in this regard have focused on pathogenic bacteria (Gevers et al., 2003; Rizzotti et al., 2005), and data on AR in lactobacilli are relatively limited (Jacobsen et al., 2007). Nevertheless, the number of studies on LAB has increased recently due to the increasing interest in probiotic bacteria and genetic modification of LAB for different purposes (Mathur and Sing, 2005; Ouoba et al., 2008). To our knowledge, ours is the first study that combines microbiological counts, the identification of antibiotic resistant LAB from the production line of a fermented dry sausage, and the screening of AR genes isolated from these bacteria. We isolated 426 *Lactobacillus* colonies, comprising 92 different strains.

350 *L. sakei* and *L. curvatus* are the species of LAB most adapted to meat
351 fermentation processes (Rantsiou and Cocolin, 2006, Urso et al., 2006).
352 Our study showed that *L. plantarum* and *L. sakei* were the AR species
353 most frequently found along the process line of fermented sausage. In
354 fact, *L. plantarum* can be an important participant in sausage
355 fermentation (Rantsiou and Cocolin, 2006, Drosinos et al., 2007).
356 However, only a few lactobacilli belonging to *L. curvatus* were isolated
357 in this study, possibly because of the type of ingredients, the
358 manufacturing process, or the ripening conditions. In fact, neither
359 Samelis et al. (1998) nor Coppola et al. (1998) isolated *L. curvatus*
360 during their studies. *L. reuteri* was the prevalent species isolated from
361 swine stool samples, as has been reported by Korhonen et al. (2007).
362 Less than 10^2 cfu/g were isolated from the minced meat and at day 0
363 in either type of antibiotic-containing media, while after 21 days the
364 number had increased significantly to 10^6cfu/g and remained stable at
365 10^6–10^7 cfu/g in tetracycline and erythromycin medium, respectively,
366 until the 45th day of ripening. In fact, LAB are usually present in raw
367 meat at low numbers but they rapidly dominate fermentation due to the
368 anaerobic environment and the presence of nitrate and nitrite, conditions
369 that favour their growth (Hammes and Knauf, 1994).
370 Considering that 10^5 cfu/g erythromycin-resistant lactobacilli were
371 isolated from the casing but that there were less than 10^2 cfu/g isolated
372 from the minced meat, at least part of bacteria that occurred during the
373 ripening originated in the casing. According to REP-PCR fingerprinting,
374 31 colonies isolated from the casing belonged to four different strains (*L.*
375 *plantarum* 2, *L. sakei* 73 and 109, and *L. reuteri* 27). The former (*L.*
376 *plantarum* 2, representing 32.3% of the LAB in the casing) was also

found along the process line until 35 days of ripening, and it was the only strain found at day 0, while *L. sakei* 73 (representing 61.3% of the LAB in the casing) was also found in the end product (45 days of ripening). Because these strains were not found in the minced meat, contamination must have occurred between the casing and single points in the process line. *L. reuteri* 27 was the only strain found in the minced meat and it persisted during the entire production process. This is the same strain that was found in the casing, suggesting that contamination occurred between the casing and the minced meat steps. To our knowledge this is the first study that shows that the casing can represent a font of AR lactobacilli during the fermentation of dry sausage.

We found that 89 of 92 strains (96.7%) were phenotypically resistant to tetracycline (20 from the food chain and 69 from stools) and 61 of 92 strains (66.3%) were phenotypically resistant to erythromycin (13 from the food chain and 48 from stools). T*et(M)* and *tet(W)* were the prevalent tetracycline resistance genes, the former being detected along almost the entire process line, the latter was present mostly in stool samples. The high incidence of *tet(M)* among our tetracycline-resistance isolates is in agreement with the wide distribution of this gene among *Lactobacillus* spp. isolated from fermented dry sausages (Gevers et al., 2003) and in DNA extracted directly from pork meat (Garofalo et al., 2007). These results also indicate that the spread of tetracycline resistance genes persists despite the ban of this antibiotic as a growth promoter in the European Union (Rizzotti et al., 2005).

ErmB was the most frequently detected macrolide-lincosamide-streptogramins gene, confirming previous reports (Aquilanti et al., 2007;

403 Garofalo et al., 2007). The *ermC* gene was found only in swine stools,
404 confirming its lesser prevalence (Aquilanti et al., 2007).
405 We detected in the production chain 12 strains that were doubly
406 resistant, and seven of these harboured both *tet* and *erm* genes. Two
407 strains carried multiple *tet* genes. We detected 47 doubly resistant strains
408 from stool samples, 40 harboured both *tet* and *erm* genes and one strain
409 carried multiple *tet* genes. The simultaneous presence of *tet* and *erm*
410 genes has been described in enterococci, streptococci, and staphylococci
411 (Chopra and Roberts, 2001; Rizzotti et al., 2005). Moreover, the
412 simultaneous occurrence of *tet(M)* / *tet(W)* and *tet(M)* / *tet(S)* is in
413 agreement with the carriage by Gram-positive bacteria of multiple *tet*
414 genes that can have either the same mode of action (efflux or ribosomal
415 protection) or different modes of action (efflux and ribosomal protection)
416 (Chopra and Roberts, 2001). Recently, Simeoni et al. (2008) found that
417 72.7% of their staphylococci isolates carried two tetracycline resistance
418 determinants, underscoring the great diffusion of this type of resistance.
419 This study provides evidence of the wide occurrence of AR
420 lactobacilli in the process line of a dry fermented sausage produced in the
421 North of Italy and in swine stools. Although these AR lactobacilli could
422 serve as reservoir organisms, the amount of these drug resistant bacteria
423 per gram of product is quite low, suggesting that the estimated risk of
424 transferring these AR genes to pathogens would be low to very low.
425 Further investigations should be applied to other food production chains,
426 to other food-associated bacteria, and to the possibility of transfer of AR
427 genes in order to evaluate the health risk of the presence of AR in foods.
428 It would be beneficial to perform a follow-up study within a few
429 years to ascertain whether the incidence of AR in the food chain of

fermented meat products decreases following the ban on the use of antibiotics as growth promoters in January 1, 2006 (Regulation (EC) n° 1831/2003).

Acknowledgements

This study was funded by the Ministero delle Politiche Agricole e Forestali, project "ARAFOA – Risk assessment related to the antibiotic resistance in bacteria used for the production of fermented food (cheese and cultured meat), specially with regard to typical and PDO products". Chapter 7303, D.M. 662/7303/03 dated 23/12/2003.

457 **References**

458 Aminov, R.I., Garrigues-Jeanjean, N., Mackie, R.I., 2001.
459 Molecular ecology of tetracycline resistance: development and validation
460 of primers for detection of tetracycline resistance genes encoding
461 ribosomal protection proteins. Applied and Environmental Microbiology
462 67, 22-32.

463 Aquilanti, L., Garofalo, C., Osimani, A., Silvestri, G., Vignarolli,
464 C., Clementi, F., 2007. Isolation and molecular characterization of
465 antibiotic-resistant lactic acid bacteria from poultry and swine meat
466 products. Journal of Food Protection 70, 557-565.

467 Aymerich, T., Martín, B., Garriga, M., Vidal-Carou, M.C., Bover-
468 Cid, S., Hugas, M., 2006. Safety properties and molecular strain typing
469 of lactic acid bacteria from slightly fermented sausages. Journal of
470 Applied Microbiology 100, 40-49.

471 Casaburi, A., Di Monaco, R., Cavella, S., Toldrá, F., Ercolini, D.,
472 Villani, F., 2008. Proteolytic and lipolytic starter cultures and their effect
473 on traditional fermented sausages ripening and sensory traits. Food
474 Microbiology 25, 335-347.

475 Chopra, I., Roberts, M., 2001. Tetracycline antibiotics: mode of
476 action, applications, molecular biology, and epidemiology of bacterial
477 resistance. Microbiology and Molecular Biology Reviews 65, 232-260.

478 Cocolin, L., Manzano, M., Cantoni, C., Comi, G., 2001.
479 Denaturing gradient gel electrophoresis analysis of the 16S rRNA gene
480 V1 region to monitor dynamic changes in the bacterial population during
481 fermentation of Italian sausages. Applied and Environmental
482 Microbiology 67, 5113-5121.

Cocolin, L., Urso, R., Rantsiou, K., Cantoni, C., Comi, G., 2006. Dynamics and characterization of yeasts during natural fermentation of Italian sausages. FEMS Yeast Research 6(5), 692-701.

Coppola, R., Giagnacovo, B., Iorizzo, M., Grazia, L., 1998. Characterization of lactobacill involved in the ripening of soppressata molisana, a typical southern Italy fermented sausage. Food Microbiology 15, 347-353.

Coppola, S., Mauriello, G., Aponte, M., Moschetti, G., Villani, F., 2000. Microbial succession during ripening of Naples-type salami, a southern Italian fermented product. Meat Science 56, 321-329.

Danielsen, M., Wind, A., 2003. Susceptibility of *Lactobacillus* spp. to antimicrobial agents. International Journal of Food Microbiology 82, 1-11.

Donabedian, S.M., Thal, L.A., Hershberger, E., Perri, M.B., Chow, J.W., Bartlett, P., Jones, R., Joyce, K., Rossiter, S., Gay, K., Johnson, J., Mackinson, C., Debess, E., Madden, J., Angulo, F., Zervos, M.J., 2003. Molecular characterization of gentamicin-resistant enterococci in the United States: evidence of spread from animals to humans through food. Journal of Clinical Microbiology 41, 1109-1113.

Drosinos, E.H., Paramithiotis, S., Kolovos, G., Tsikouras, I., Metaxopoulos, I., 2007. Phenotypic and technological diversity of lactic acid bacteria and staphylococci isolated from traditionally fermented sausages in Southern Greece. Food Microbiology 24, 260-270.

EFSA, 2005. Opinion of the Scientific Panel on additives and products or substances used in animal feed on the updating of the criteria used in the assessment of bacteria for resistance to antibiotics of human or veterinary importance. The EFSA Journal 223, 1-12.

Fontana, C., Cocconcelli, P.S., Vignolo, G., 2005. Monitoring the bacterial population dynamics during fermentation of artisanal Argentinean sausages. International Journal of Food Microbiology 103, 131-142.

Garofalo, C., Vignaroli, C., Zandri, G., Aquilani, L., Bordoni, D., Osimani, A., Clementi, F., Biavasco, F., 2007. Direct detection of antibiotic resistance genes in specimens of chicken and pork meat. International Journal of Food Microbiology 113, 75-83.

Gevers, D., Huys, G., Swings, J., 2001. Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. FEMS Microbiology Letters 205(1), 31-36.

Gevers, D., Danielsen, M., Huys, G., Swings, J., 2003. Molecular characterization of tet(M) genes in *Lactobacillus* isolates from different types of fermented dry sausage. Applied and Environmental Microbiology 69, 1270-1275.

Hammes, W.P., Knauf, H.J., 1994. Starters in processing of meat products. Meat Science 36, 155-168.

Jacobsen, L., Wilcks, A., Hammer, K., Huys, G., Gevers, D., Andersen, S.R., 2007. Horizontal transferof tet(M) and erm(B) resistance plasmids from food strains of *Lactobacillus plantarum* to *Enterococcus faecalis* JH2-2 in the gastrointestinal tract of gnotobiotic rats. FEMS Microbiology Ecology 59, 158-166.

Jensen, L.B., Frimodt-Moller, N., Aarestrup, F.M., 1999. Presence of *erm* gene classes in Gram-positive bacteria of animal and human origin in Denmark. FEMS Microbiology Letters 170, 151-158.

Klare, I., Konstabel, C., Müller-Bertling, S., Reissbrodt, R., Huys, G., Vancanneyt, M., Swings, J., Goossens, H., Witte, W., 2005.

Evaluation of new broth media for microdilution antibiotic susceptibility testing of Lactobacilli, Pediococci, Lactococci, and Bifidobacteria. Applied and Environmental Microbiology 71, 8982-8986.

Korhonen, J.M., Sclivagnotis, Y., von Wright, A.J., 2007. Characterization of dominant cultivable lactobacilli and their antibiotic resistance profiles from faecal samples of weaning piglets. Journal of Applied Microbiology 103, 2496-2503.

Lucke, F.K., 1985. Fermented sausages. In: Wood, B.J.B. (Ed.), Microbiology of fermented foods. Elsevier Applied Science, London, pp. 41-83.

Mathur, S., Singh, R., 2005. Antibiotic resistance in food lactic acid bacteria-a review. International Journal of Food Microbiology 105, 281-295.

Morot-Bizot, S.C., Leroy, S., Talon, R., 2006. Staphylococcal community of a small unit manufacturing traditional dry fermented sausages. International Journal of Food Microbiology 108, 210-217.

Olsvik, B., Olsen, I., Tenover, F.C., 1995. Detection of tet(M) and tet(O) using the polymerase chain reaction in bacteria isolated from patients with periodontal disease. Oral Microbiology and Immunology 10, 87-92.

Ouoba, L.I.I., Lei, V., Jensen, L.B., 2008. Resistance of potential probiotic lactic acid bacteria and bifidobacteria of African and European origin to antimicrobials: Determination and transferability of the resistance genes to other bacteria. International Journal of Food Microbiology 121, 217-224.

Parente, E., Greco, S., Crudele, M.A., 2001. Phenotypic diversity of lactic acid bacteria isolated from fermented sausages produced in

564 Basilicata (Southern Italy). Journal of Applied Microbiology 90, 943-
565 952.

566 Phillips, I., Casewell, M., Cox, T., De Groot, B., Friis, C., Jones,
567 R., Nightingale, C., Preston, R., Waddell, J., 2004. Does the use of
568 antibiotics in food animals pose a risk to human health? A critical review
569 of published data. Journal of Antimicrobial Chemotherapy 53, 28-52.

570 Poyart, C., Jardy, L., Quesne, G., Berche, P., Trieu-Cuot, P.,
571 2003. Genetic basis of antibiotic resistance in *Streptococcus agalactiae*
572 strains isolated in a French hospital. Antimicrobial Agents and
573 Chemotherapy 47, 794-797.

574 Rantsiou, K., Cocolin, L., 2006. New developments in the study
575 of the microbiota of naturally fermented sausages as determined by
576 molecular methods: a review. International Journal of Food Microbiology
577 108, 255-267.

578 Regulation (EC) n° 1831/2003 (http://eur-
579 lex.europa.eu/en/index.htm).

580 Rizzotti, L., Simeoni, D., Cocconcelli, P., Gazzola, S., Dellaglio,
581 F., Torriani, S., 2005. Contribution of enterococci to the spread of
582 antibiotic resistance in the production chain of swine meat commodities.
583 Journal of Food Protection 68, 955-965.

584 Rowe-Magnus, D.A., Guerout, A.M., Ploncard, P., Dychinco, B.,
585 Davies, J., Mazel, D., 2001. The evolutionary history of chromosomal
586 super-integrons provides an ancestry formultiresistant integrons.
587 Proceedings of the National Academy of Sciences of the United States of
588 America 98, 652-657.

589 Saarela, M., Maukonen, J., von Wright, A., Vilpponen-Salmela,
590 T., Patterson, A.J., Scott, K.P., Hämynen, H., Mättö, J., 2007.

Tetracycline susceptibility of the ingested *Lactobacillus acidophilus* LaCH-5 and *Bifidobacterium animalis* subsp. *lactis* Bb-12 strains during antibiotic/probiotic intervention. International Journal of Antimicrobial Agents 29, 271-280.

Samelis, J., Metaxopoulos, J., Vlassi, M., Pappa, A., 1998. Stability and safety of traditional Greek salami-a microbiological ecology study. International Journal of Food Microbiology 23, 179-196.

Simeoni, D., Rizzotti, L., Cocconcelli, P., Gazzola, S., Dellaglio, F., Torriani, S., 2008. Antibiotic resistance genes and identification of staphylococci collected from the production chain of swine meat commodities. Food Microbiology 25, 196-201.

Sorensen, T.L., Blom, M., Monnet, D.L., Frimodt-Moller, N., Poulsen, R.L., Espersen, F., 2001. Transient intestinal carriage after ingestion of antibioticresistant *Enterococcus faecium* from chicken and pork. New England Journal of Medicine 345, 1161-1166.

Teuber, M., Meile, L., Schwarz, F., 1999. Acquired antibiotic resistance in lactic acid bacteria from food. Antonie Leeuwenhoek 76, 115-137.

Teuber, M., Perreten, V., 2000. Role of milk and meat products as vehicles for antibiotic-resistant bacteria. Acta Veterinaria Scandinavica Supplementum 93, 75-87.

Urso, R., Comi, G., Cocolin, L., 2006. Ecology of lactic acid bacteria in Italian fermented sausages: isolation, identification and molecular characterization. Systematic and Applied Microbiology 29(8), 671-680.

Ventura, M., Casas, I.A., Morelli, L., Callegari, M.L., 2000. Rapid amplified ribosomal DNA restriction analysis (ARDRA)

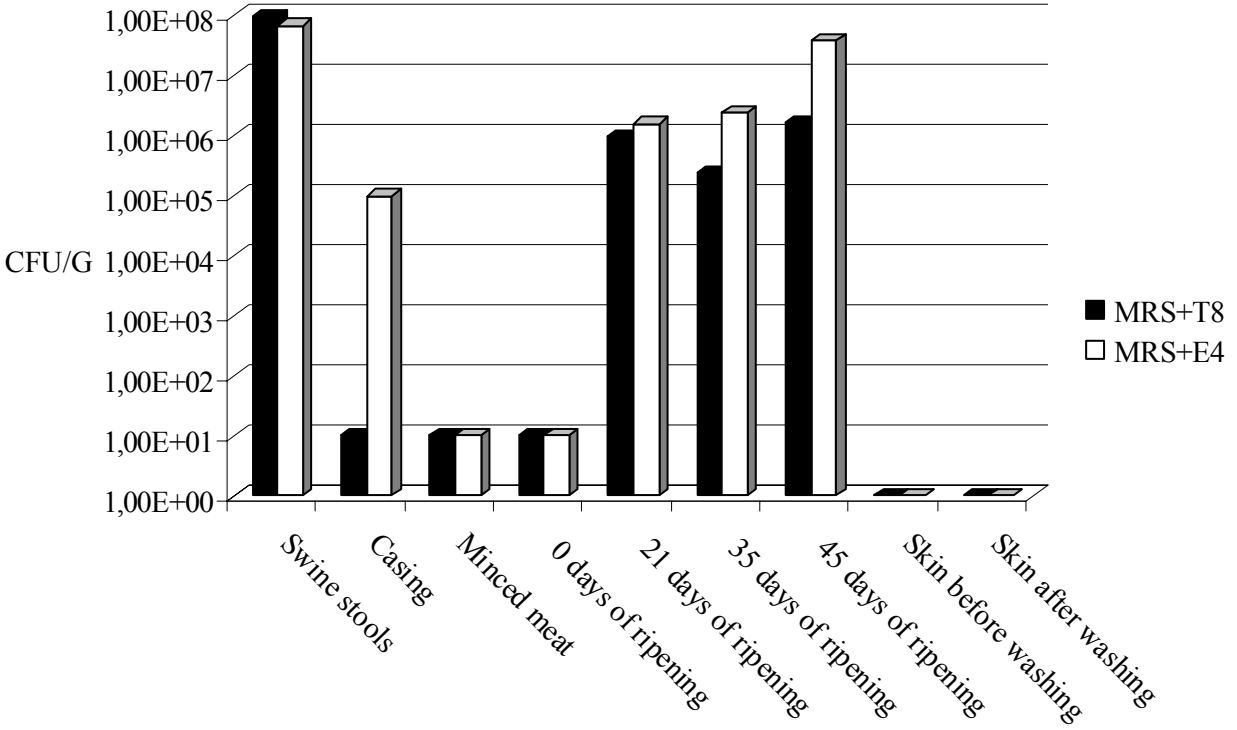
identification of *Lactobacillus* spp. isolated from faecal and vaginal samples. Systematic and Applied Microbiology 23, 504-509.

Villani, F., Casaburi, A., Pennacchia, C., Filosa, L., Russo, F., Ercolini, D., 2007. Microbial ecology of the soppressata of Vallo di Diano, a traditional dry fermented sausage from southern Italy, and in vitro and in situ selection of autochthonous starter cultures. Applied and Environmental Microbiology 73, 5453-5463.

Legends to figures:

Figure 1. Microbial counts of the tetracycline- and erythromycin-resistant lactobacilli from swine stools and from the production chain of an Italian salami

662 Figure 1. Microbial counts of the tetracycline- and erythromycin-resistant lactobacilli from swine stools and
663 from the production chain of an Italian salami
664



665

666 Table 1. Primers used for PCR-based detection of antibiotic resistance genes

Primer pair	Target gene	Sequence (5'–3')	Amplicon size (bp)	Reference	Positive control strains
ermB	*ermB*	GGTAAAGGGCATTTAACGAC	454	Poyart *et al* 2003	*L. sakei 13*[a]
ermB		CGATATTCTCGATTGACCCA			
ermC-1	*ermC*	ATCTTTGAAATCGGCTCAGG	294	Jensen *et al* 1999	*L. reuteri* 70[a]
ermC-2		CAAACCCGTATTCCACGATT			
tetM-1	*tet(M)*	GAACTCGAACAAGAGGAAAGC	740	Olsvik *et al* 1995	*L. plantarum* 30[a]
tetM-2		ATGGAAGCCCAGAAAGGAT			
tetL-FW	*tet(L)*	GTMGTTGCGCGCTATATTCC	696	Gevers *et al* 2003	*E. faecium* LMG20927[b]
tetL-RV		GTGAAMGRWAGCCCACCTAA			
tetW-FW	*tet(W)*	GAGAGCCTGCTATATGCCAGC	168	Aminov *et al* 2001	*Bifidobacterium animalis* subsp. *lactis* Bb-12[C]
tetW-RV		GGGCGTATCCACAATGTTAAC			
tetS-FW	*tet(S)*	GAAAGCTTACTATACAGTAGC	169	Aminov *et al* 2001	*L. plantarum* 31[a]
tetS-RV		AGGAGTATCTACAATATTTAC			

667 [a] Collection of microorganisms of the Microbiology Institute, Università Cattolica del Sacro Cuore, Piacenza (Italy).

668 [b] BCCM/LMG, Bacteria Collection, Belgium

669 [c] Saarela *et al* 2007

670

671

672

673 Table 2. Diversity among the lactobacilli (n = 426) isolated from swine stools and from the production chain of an

674 Italian salami

	Total number of isolates (%)	Swine stools	Casing	Minced meat	Skin before washing	Skin after washing	0 days[a]	21 days[a]	35 days[a]	45 days[a]
L. reuteri	189 (44.4)	150	1	3	-	-	1	4	17	14
L. plantarum	88 (20.7)	15	10	-	-	-	20	11	19	13
L. sakei	79 (18.5)	4	20	-	-	-	-	14	2	39
L. paracasei	21 (4.9)	-	-	-	-	-	-	15	2	4
L. amylovorus	16 (3.8)	16	-	-	-	-	-	-	-	-
L. brevis	16 (3.8)	14	-	-	-	-	-	1	-	1
L. fermentum	9 (2.1)	10	-	-	-	-	-	-	-	-
L. johnsonii	4 (0.9)	4	-	-	-	-	-	-	-	-
L. curvatus	4 (0.9)	-	-	-	-	-	-	-	4	-
Total	**426**	**213**	**31**	**3**	**-**	**-**	**21**	**45**	**44**	**71**

675 [a] Days of ripening

676

677

678

679

Table 3. MIC data for *Lactobacillus* species determined in LSM broth by microdilution

Antimicrobials	Species (n° of isolates tested)	MIC range (μg/ml)	MIC50 (μg/ml)	MIC90 (μg/ml)
Erythromycin	*L. reuteri* (44)	0.25-512	64	512
	L. plantarum (11)	0.25-512	2	64
	L. sakei (10)	0.25-128	2	128
	L. paracasei (3)	0.25-512	4	512
	L. amylovorus (8)	0.25-512	2	512
	L. brevis (6)	0.25-128	32	128
	L. fermentum (6)	2-512	128	512
	L. johnsonii (2)	128	128	128
	L. curvatus (2)	4-32	4	32
Tetracycline	*L. reuteri* (44)	128-512	512	512
	L. plantarum (11)	16-512	32	512
	L. sakei (10)	16-256	32	128
	L. paracasei (3)	32-512	32	512
	L. amylovorus (8)	128-512	128	512
	L. brevis (6)	16-512	16	512
	L. fermentum (6)	256-512	256	512
	L. johnsonii (2)	256	256	256
	L. curvatus (2)	32-512	32	512

706 Table 4. Erythromycin and tetracycline resistance in *Lactobacillus* strains from the swine stools and from the
707 production chain

Antibiotic resistance	Casing	Minced meat	Skin before washing	Skin after washing	0 days[a]	21 days[a]	35 days[a]	45 days[a]	Stools
Total strain number	4	1	0	0	2	8	9	12	70
Erythromycin resistant strains	2	1	0	0	0	4	7	7	48
ermB	1	1	0	0	0	2	6	5	44
ermC	0	0	0	0	0	0	0	0	3
Tetracycline resistant strains	3	1	0	0	1	7	8	10	69
tet(M)	2	0	0	0	1	4	6	5	16
tet(L)	0	0	0	0	0	0	0	0	0
tet(S)	1	0	0	0	1	1	2	0	1
tet(W)	1	1	0	0	1	1	2	1	45

708 [a] Days of ripening

709

710

711

712

713 Table 5. Erythromycin and tetracycline resistance in lactobacilli

Lactobacillus species	Total strain number	Erythromycin resistant strains	*ermB*	*ermC*	Tetracycline resistant strains	*tet(M)*	*tet(L)*	*tet(S)*	*tet(W)*	Double resistance	*ermB* + *tet(M)*	*ermB* + *tet(W)*	*ermB* + *tet(S)*	*ermC* + *tet(W)*	Multiple *tet*
L. reuteri	44	32	29	2	44	7	0	0	31	32	5	20		2	
L. plantarum	11	4	2	0	8	5	0	3	1	2	1		1		2
L. sakei	10	5	4	0	10	4	0	0	2	5	1	2			1
L. paracasei	3	2	1	0	3	3	0	0	0	2	1				
L. amylovorus	8	4	3	1	8	0	0	0	7	4		3		1	
L. brevis	6	5	5	0	5	5	0	0	1	5	4	1			1
L. fermentum	6	5	5	0	6	2	0	0	4	5	2	3			
L. johnsonii	2	2	2	0	2	0	0	0	1	2		1			
L. curvatus	2	2	2	0	2	2	0	0	1	2	2	1			1
Total	**92**	**61**	**53**	**3**	**89**	**28**	**0**	**3**	**48**	**59**	**16**	**31**	**1**	**3**	**5**

714

715

716

717

718

719

2.2. ERYTHROMYCIN AND TETRACYCLINE RESISTANT LACTOBACILLI IN ITALIAN FERMENTED DRY SAUSAGES

This paper was submitted to the "*Journal of Applied Microbiology*" and is still subject to approval for publication

Erythromycin and tetracycline resistant lactobacilli in Italian fermented dry sausages

Zonenschain Daniela[1,*], Rebecchi Annalisa[2], Morelli Lorenzo[1,2]

[1] Istituto di Microbiologia, Facoltà di Agraria, Università Cattolica del Sacro Cuore, Via Emilia Parmense 84, 29100 Piacenza, Italy

[2] Centro Ricerche Biotecnologiche, Via Milano 24, 26100 Cremona, Italy

Running headline: Antibiotic resistance in lactobacilli

** Correspondence to: Daniela Zonenschain, Istituto di Microbiologia, Facoltà di Agraria, U.C.S.C., Via Emilia Parmense 84, 29100, Piacenza, Italy Tel.: +39 0523 599244; fax: +39 0523 599246. E-mail address: daniela.zonenschain@unicatt.it*

Abstract

Aims: To assess the frequency of erythromycin and tetracycline resistant lactobacilli in Italian fermented dry sausages.

Methods and Results: We isolated from 20 salami from the north of Italy (Piacenza province) colonies of lactobacilli from selective medium supplemented with erythromycin or tetracycline, determined the minimum inhibitory concentration of *Lactobacillus* isolates and screened selected erythromycin and tetracycline resistance genes. A total of 312 colonies of lactobacilli were genetically ascribed to 60 different strains belonging to seven *Lactobacillus* species. *Lactobacillus sakei*, *Lactobacillus curvatus* and *Lactobacillus plantarum* were the most frequently found species. Thirty (50%) strains were phenotypically resistant to erythromycin, 45 (75%) to tetracycline, and 27 (45%) were resistant to both. The most frequently detected resistance genes were *tet*(M) and *erm*(B).

Conclusions: This study provides evidence of the presence of tetracycline and, to a lesser extent, erythromycin resistant lactobacilli in fermented dry sausages produced in northern Italy.

Significance and Impact of Study: Although these antibiotic resistant lactobacilli could serve as reservoir organisms, in our study 80% of salami could be considered as safe even though 20% could represent a border line situation regarding the possibility of transferring AR genes to pathogens.

Keywords: Antibiotic resistance, erythromycin, fermented dry sausages, *Lactobacillus*, tetracycline

Introduction

Fermented sausages are a traditional product with great diversity in production methods and organoleptic characteristics between different countries and different regions of the same country (Rantsiou et al. 2005). Multiple kinds of fresh and fermented sausages are produced in Italy, many of which are only marketed locally (Comi et al. 2005).

The qualitative characteristics of naturally fermented sausages are largely dependent on the quality of the ingredients and raw materials, the specific conditions of the processing and ripening, and the composition of the microbial population (Aquilanti et al. 2007). Control during processing is essential in terms of the microbiological quality, sensory characteristics of the final product and food safety (Talon et al. 2008). Several studies have shown that the microbiota of these products mainly consist of lactic acid bacteria (LAB) and coagulase negative cocci (CNC) (Rantsiou and Cocolin 2006), followed by enterococci and, to a lesser extent, yeasts and molds (Villani et al. 2007).

Many LAB species are involved in the manufacture and preservation of fermented feed and foods from raw agricultural materials (such as milk, meat, vegetables and cereals) in which they are present as contaminants or deliberately added as starters to control the fermentation process. These species, therefore, have a great economic importance in the food industry (Leroy and de Vuyst 2004).

The primary contribution of the LAB to flavour generation is due to their production of organic acids and volatiles through the fermentation of carbohydrates (Urso et al. 2006). Their ability to lower the pH of the mixture by producing acid from sugars leads to the development of the desirable organoleptic properties, prevents the growth

of pathogens and ensures the stability and safety of the final product (Lucke 1985).

Coagulase-negative staphylococci participate in colour stabilization, decomposition of peroxide, reduction of nitrates to nitrites (Iacumin et al. 2006) and aroma formation due to their proteolytic and lipolytic activities (Miralles et al. 1996).

The development of molecular methods has confirmed the presence of *Lactobacillus sakei*, *Lactobacillus curvatus* and *Lactobacillus plantarum* as the most commonly identified LAB species in traditional fermented sausages (Coppola et al. 2000; Aymerich et al. 2006; Urso et al. 2006). Among CNC isolates, *Staphylococcus xylosus* is frequently isolated as the main species, but others have also been reported: *Staphylococcus carnosus*, *Staphylococcus simulans*, *Staphylococcus saprophyticus*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus warneri*, and *Staphylococcus equorum* (Coppola et al. 2000).

The introduction of antimicrobial agents in human clinical medicine and animal husbandry has been one of the most significant achievements of the twentieth century (Aarestrup 2005). However, antibiotic resistance (AR) in microorganisms has now become a serious medical problem, primarily attributed to the overuse of antibiotics (Egervärn et al. 2007). One concern is that the use of antibiotics in the food chain, mainly in food-producing animals, has contributed to the development and spread of resistant bacteria in the environment (Tenover and Hughes 1996).

AR in LAB has garnered increasing attention in recent years (Danielsen and Wind 2003; Flórez et al. 2005; Gevers et al. 2003a).

Because of their broad environmental distribution, LAB may function as reservoirs of antibiotic resistance genes that can be transferred via the food chain or within the gastrointestinal tract to other bacteria, including human pathogens (Teuber et al. 1999).

AR in pathogenic bacteria has been a medical problem for decades, though recently, resistance determinants have been also found to be widespread among isolates from non-clinical settings. Staphylococci, as well as enterococci and other LAB, which are omnipresent members of the intestinal flora, have been isolated both from food and intestinal samples and shown to carry antibiotic resistance determinants (Flórez et al. 2005; Huys et al. 2004).

Bacteria involved in food fermentation may be AR reservoirs (Danielsen and Wind 2003). Raw meat and fermented foods are therefore potential vehicles for the spread of antibiotic-resistant bacteria and/or AR to pathogens and ultimately to the consumer (Sorensen et al. 2001), raising major concerns with regard to food safety. Therefore, food products containing commensal bacteria resistant to antibiotics could be considered potential vehicles for AR genes that can be spread to pathogens (Danielsen and Wind 2003).

Nevertheless, the administration of antibiotics to animals can select antibiotic resistant species, depending on the spectrum of activity of the antimicrobial agents (Teale 2002). As a consequence, an emerging reservoir of antibiotic resistant microbes could occupy the niches of antibiotic sensitive species or spread resistance genes to other microorganisms via horizontally mobile genetic elements, such as viruses, plasmids, and transposons (Heinemann et al. 2000).

The aim of this study was to identify tetracycline and erythromycin resistant *Lactobacillus* colonies isolated from 20 Italian fermented sausages (Piacentino salami) at the species and strain level, and to evaluate the diffusion of AR genes in these isolates.

Materials and methods

Fermented sausage technology and sampling procedures

The Piacentino salami is a fermented Italian dry sausage produced in north Italy (Piacenza province) without the use of starter cultures. It is manufactured according to the traditional technique, using pork meat and the following ingredients: lard (25%), salt (25 g kg^{-1}), black pepper (4.0 g kg^{-1}), white wine (5.0 ml kg^{-1}), crushed garlic (2.0 g kg^{-1}), nitrate, and ascorbic acid. The batter is stuffed into natural casings and ripened as follows: one week of fermentation under relative humidity (RH) ranging from 40-90% at 15-25°C and six weeks of drying at 70-90% RH and 12-19°C. The final product is presented in cylindrical form and weighs between 0.4 and 1 kg. For commercial sale, the product must receive the pertinent Protected Designation of Origin (PDO) seal, which attests to its origin and traditional production practices.

The samples analyzed in this study were obtained from 20 artisanal factories producing Piacentino salami and were collected after 45 days of ripening.

Microbiological analysis

After aseptically removing the casing, we transferred 25 grams of each sample into a sterile stomacher bag and added 225 ml of saline peptone water (8 g l^{-1} of NaCl, 1 g l^{-1} of bacteriological peptone, Oxoid).

The preparation was mixed in a stomacher apparatus (400 Circulator, PBI, Milan, Italy) at 260 rpm for 2 min.

Decimal dilutions of the homogenates were prepared using the same diluents, plated on de Man, Rogosa, Sharpe (MRS) agar (Oxoid), and incubated at 30°C in anaerobiosis for 48 h. Growth medium was supplemented with 4 μg ml^{-1} of erythromycin (Sigma) or 8 μg ml^{-1} of tetracycline (Sigma) to screen for antibiotic resistant lactobacilli. Concentrations of antibiotics were based on the breakpoints values defined by European Food Safety Authority (EFSA, 2005).

The colonies on each plate were counted (the detection limit was 10^2 g^{-1}) and 10% (3-30) lactobacilli colonies were randomly selected, streaked on MRS agar plates, and subcultured in tubes containing MRS supplemented with the antimicrobial agent at the same concentration used for the initial isolation. The antibiotic resistant isolates were purified and stored at -80°C in 25% glycerol before molecular analysis.

DNA extraction from pure cultures

Four millilitres of a 24 h culture were centrifuged at 14,000g for 10 min at 4°C to pellet the cells, which were subjected to DNA extraction using the Puregene DNA Purification Kit (Gentra Systems, Minneapolis, USA) following the manufacturer's instructions.

Species identification

Amplified Ribosomal DNA Restriction Analysis (ARDRA) was performed as described by Ventura et al. (2000) to identify species of *Lactobacillus*. Briefly, the 16S rRNA gene was amplified by PCR using the P0 (5'- GAG AGT TTG ATC CTG GCT- 3') and P6 (5'- CTA CGG

CTA CCT TGT TAC - 3') primers. The amplification reaction was performed in a total volume of 25 µL that contained 10 ng DNA, 0.5 µM of each primer and the Megamix (Labogen). The initial denaturation was performed at 94 °C for 3 min, followed by 30 cycles of 94 °C for 45 s, 55 °C for 45 s and 72 °C for 60 s, and a final extension at 72 °C for 7 min. The PCR was carried out in a Gene Amp 9700 thermal cycler (Applied Biosystem, Foster City, USA. The amplification products were subjected to gel electrophoresis in a 0.8 % agarose gel at 100 V for 30 min, followed by ethidium bromide staining. The amplified 16S rDNA was digested with restriction enzymes *Sau*3AI, *Hin*fI, *Dra*I or *Hinc*II (Roche Diagnostics GmbH, Basel, Switzerland) and the products were subjected to electrophoresis in a 3% (w/v) agarose gel at 120 V for 2–3 h, followed by ethidium bromide staining.

To confirm species identification, PCR products from one representative of each species were purified using the Wizard SV Gel and PCR Clean-Up system according to the package insert (Promega Corporation, Madison, Wis., USA) and sequenced at the Biomolecular Research (BMR) Centre, University of Padova, Italy. The identities of the isolates were determined by comparison against sequences in the GenBank DNA database (http://www.ncbi.nlm.nih.gov/).

Strain typing

Repetitive Extragenic Palindromic (REP) PCR using the $(GTG)_5$ primer was used to identify lactobacilli isolates at the strain level as previously described by Gevers et al. (2001). The amplification reaction was performed in a total volume of 25 µL that contained 10 ng DNA, 0.5 µM primer and the Megamix (Microzone Limited, UK). The PCR was

carried out in a Gene Amp 9700 thermal cycler (Applied Biosystem, Foster City, USA) as follows: initial denaturation was performed at 95 °C for 7 min, followed by 30 cycles of 90 °C for 30 s, 40°C for 1 min and 65 °C for 8 min, and a final extension at 65 °C for 16 min. PCR products were analyzed on a 2% agarose gel at 80 V (Bio-Rad Laboratories, Milan, Italy) gels, and a 200 bp ladder (Promega Corporation, Madison, Wis., USA) was included for molecular weight standards. The gel was subsequently stained with 0.5 μg ml^{-1} ethidium bromide. The fingerprinting patterns were analyzed using Gel Compare 4.0 software (Applied Math, Kortrijk, Belgium).

Antibiotic susceptibility testing

The phenotypic antimicrobial resistance of a strain to a certain antibiotic was determined as the minimum inhibitory concentration (MIC), defined as the lowest antibiotic concentration that resulted in no visible growth. MICs were determined by the broth microdilution method using the standardized LAB susceptibility test medium (LSM) broth formulation, which ensures adequate growth of the test organisms; LSM essentially consists of a mixture of Iso-Sensitest broth medium (Oxoid) (90%) and MRS broth medium (10%) adjusted to pH 6.7 as previously described by Klare et al. (2005).

Tetracycline was tested at 4-512 μg ml^{-1}, and erythromycin was tested at 0.25-512 μg ml^{-1}. Bacteria were inoculated into LSM broth to a final concentration of 3×10^5 cfu ml^{-1} and incubated at 37°C for 48 h in anaerobiosis.

MIC50 and MIC90 are defined as the MIC that inhibits 50% and 90% of the isolates tested, respectively; these were determined for the above-mentioned antimicrobials for all strains tested in this study.

PCR detection of antimicrobial resistance genes

The presence of genes involved in resistance to tetracycline (*tet*(L), *tet*(M), *tet*(S), *tet*(W)) and macrolide-lincosamide-streptogramins (*erm*(B), *erm*(C)) was determined by PCR. Approximately 10 ng of bacterial DNA was used for PCR in a total volume of 25 µl containing 0.5 µM of each primer and Megamix (Microzone Limited, UK). Positive control DNA was included in each PCR reaction, and a negative control reaction containing no template was included in each run. Primer pair sequences, target genes, amplicon sizes, reference strains used as positive controls and PCR protocol references are listed in Table 1.

To confirm the results, PCR products of each AR gene were selected at random, purified using the Wizard SV Gel and PCR Clean-Up system according to the manufacturer's protocol (Promega Corporation, Madison, Wis., USA), and sequenced at the BMR Centre. The BlastN program was used for nucleotide sequence analysis.

Results

Analysis and quantification of lactobacilli

We analyzed samples of 20 PDO fermented dry sausages after 45 days of ripening for the presence of erythromycin and tetracycline resistant lactobacilli. The colony counts are shown in Figure 1. Using selective medium without antibiotics, samples from 14 out of the 20 salami presented counts of approximately 10^7-10^8 cfu g^{-1}, four samples

presented approximately 10^5-10^6 cfu g^{-1}, while the remaining two salami presented 10^3 cfu g^{-1}. On tetracycline plates, samples from five of 20 salami presented approximately 10^6-10^7 cfu g^{-1}, six presented counts of about 10^4-10^5 cfu g^{-1}, six showed counts of 10^2 cfu g^{-1}, and the remaining three showed $<10^2$ cfu g^{-1}. On erythromycin plates, only one of 20 samples presented 10^7 cfu g^{-1}, four presented counts of 10^3-10^4 cfu g^{-1}, 12 out of 20 salami presented counts of 10^2 cfu g^{-1}, while the remaining three showed $<10^2$ cfu g^{-1}.

Species identification

A total of 312 colonies of lactobacilli were isolated from media with antibiotics and seven different species were detected: *Lact. sakei,* *Lact. curvatus*, *Lact. plantarum*, *Lactobacillus brevis*, *Lactobacillus rhamnosus*, *Lactobacillus paracasei* and *Lactobacillus reuteri*. The first three species cited above were the most frequently found (Figure 2). Of the total 312 isolates, 101 were *Lact. sakei,* and this species was found in 10 out of 20 salami; 94 isolates were confirmed as *Lact. curvatus*, and were distributed in 12 out of 20 salami, and 80 isolates were identified as *Lact. plantarum* and they were found in 7 out of 20 salami.

In regard to the diversity of species, one salami presented five different antibiotic resistant *Lactobacillus* species, five salami presented three or four different species, 12 salami presented one or two species, and two salami did not present any antibiotic resistant *Lactobacillus* isolates.

Strain typing

REP pattern analysis demonstrated the presence of 60 different strains of *Lactobacillus* distributed as follows: 24 strains of *Lact. sakei,* 16 *Lact. curvatus*, 12 *Lact. plantarum*, three *Lact. paracasei*, two *Lact. brevis*, two *Lact. rhamnosus* and one *Lact. reuteri*. One salami presented 10 different antibiotic resistant strains, three salami presented nine different antibiotic resistant strains, one presented eight different antibiotic resistant strains, six presented between three and six different antibiotic resistant strains, seven presented one or two different antibiotic resistant strains and two salami samples did not present antibiotic resistant strains.

Determination of phenotypic antimicrobial resistance

We determined MIC values for both antibiotics for the 60 strains analyzed in this study. The MICs for tetracycline ranged between 2 and 512 μg ml^{-1}, while the erythromycin MICs ranged between 0.25 and 1024 μg ml^{-1}. The MIC values for the two antimicrobial agents tested for all strains are shown in Table 2.

Using the EFSA (2005) breakpoints reference values, strains were considered to be phenotypically resistant when the MIC of tetracycline reached 32 μg ml^{-1} for *Lact. plantarum* or 8 μg ml^{-1} for the other lactobacilli, and the MIC of erythromycin reached 4 μg ml^{-1}. According to these criteria, 30 (50%) strains were phenotypically resistant to erythromycin, 45 (75%) to tetracycline, and 27 (45%) were resistant to both. Regarding the three most common species, resistance to tetracycline and erythromycin was detected in 91% and 50% of *Lact. plantarum*, 70% and 29% of *Lact. sakei* and 62% and 62% of *Lact.*

322 *curvatus* strains, respectively. The numbers of phenotypic antimicrobial
323 resistant lactobacilli are listed in Table 3.
324 In regard to the prevalence of tetracycline resistance in the 20
325 analyzed salami, 10 samples showed all strains resistant to tetracycline,
326 at least 50% of the strains were resistant in six samples, and only three
327 salami did not contain tetracycline resistant strains. Regarding resistance
328 to erythromycin, all strains were resistant in five out of 20 salami, at least
329 50% of the strains were resistant in nine samples, and only three salami
330 did not contain erythromycin resistant strains. Four out of 20 salami
331 contained all double resistant strains, at least 50% of the strains were
332 double resistant in six samples, and five salami did not contain any
333 double resistant strains. The distribution of phenotypic antibiotic resistant
334 lactobacilli is shown in Table 4.
335
336 *PCR detection of antimicrobial resistance genes*
337 The most common tetracycline-resistance gene detected among
338 *Lactobacillus* species was *tet*(M), which was identified in 60% of the
339 tetracycline resistant strains. It was present in all species except for *Lact.*
340 *rhamnosus,* and identified in 70% of *Lact. curvatus*, 64% of *Lact. sakei*
341 and 45% of *Lact. plantarum* tetracycline-resistant strains. The *tet*(W)
342 determinant was found in 22% of the resistant strains, particularly in
343 *Lact. curvatus* (40%) and in *Lact. plantarum* (36%), while *tet*(S) was
344 found only in one *Lact. plantarum* strain and *tet*(L) was not detected. The
345 PCR analysis of the AR genes in lactobacilli strains is reported in Table
346 3.
347 Regarding the erythromycin resistant strains, *erm*(B) was the
348 most commonly found erythromycin-resistance gene, identified in 76%

of the strains. It was found in all species, and particularly in 100% of *Lact. curvatus*, 71% of *Lact. sakei* and 50% of *Lact. plantarum*. The *erm*(C) gene was only detected in *Lact. plantarum* strains. In the 27 double resistant strains, 59% presented one *erm* and one *tet* gene, and 18% presented multiple *tet* genes.

The number of AR genes found in each salami is shown in Table 4. The *tet*(M) gene was detected in 13 out of 20 salami, *tet*(W) in 10 and *tet*(S) in two salami; the *erm*(B) gene was found in 17 out of 20 salami while *erm*(C) was detected in only two salami. The presence of *erm* and *tet* genes was detected in 14 out of 20 salami while the presence of multiple *tet* determinants was found in two salami.

Discussion

The extensive use of antibiotics for treating microbial infections in humans, animals, and plants and as growth promoters in animal feed has led to the spread of AR in commensal microorganisms, creating large reservoirs of AR genes in non-pathogenic bacteria that are linked to the food chain (Aquilanti et al. 2007). These genes could potentially be transferred both horizontally and vertically; however, the implications of these findings with regard to public health remain unclear (Phillips et al. 2004). Nevertheless, the food chain is recognized as one of the main routes for the transmission of AR between animal and human populations (Teuber et al. 1999).

Most investigations in this regard have focused on pathogenic bacteria (Gevers et al. 2003a; Rizzotti et al. 2005), and data on AR in lactobacilli are relatively limited (Jacobsen et al. 2007). However, the number of studies on LAB has increased recently due to growing interest

376 in probiotic bacteria and genetic modification of LAB for various
377 purposes (Ouoba et al. 2008). Accordingly, the present study was
378 designed to evaluate the incidence of tetracycline and erythromycin
379 resistant *Lactobacillus* and a few selected AR genes in lactobacilli
380 isolated from fermented dry sausages that could constitute a significant
381 route for the spread of resistance to clinically important antibiotics.
382 The high number of salami (14 out of 20) with high lactobacilli
383 counts (approximately 10^7-10^8 cfu g^{-1}) is in agreement with reports from
384 other authors (Parente et al. 2001, Rantsiou et al. 2005, Aquilanti et al.
385 2007). In fact, LAB are usually present in raw meat at low numbers but
386 they rapidly dominate in fermentation due to the anaerobic environment
387 and the presence of nitrate and nitrite, conditions that favour their growth
388 (Hammes and Knauf 1994).
389 We isolated 312 *Lactobacillus* colonies, and these isolates were
390 identified by means of ARDRA. As has been reported, *L. sakei, L.*
391 *curvatus*, *L. plantarum* were confirmed as the species that are most
392 frequently recovered from meat products, especially in dry fermented
393 sausages (Parente et al. 2001, Urso et al. 2006), with *L. sakei* being the
394 most frequently isolated species (Urso et al. 2006).
395 In this study we found a high incidence of salami presenting
396 tetracycline - resistant isolates (17 out of 20) and a high percentage of
397 tetracycline-resistant strains in confront to the total number of strains, as
398 we can see by the fact that 10 out of 20 salami presented 100 % of strains
399 resistant to tetracycline. In the 10 salami with tetracycline-resistant
400 strains, the microbiological counts were low (from 10^2 to 10^5 cfu g^{-1}),
401 with the exception of two salami that showed counts of 10^6 and 10^7 cfu g^{-1}
402 .

Genetic determinants for tetracycline resistance were found in 14 out of 17 salami with resistant strains; *tet*(M) was the most common gene, harboured by 13 salami and representing 60% of the tetracycline resistant strains. Additionally, *tet*(W) was detected in a high number of salami (12), representing 28% of the tetracycline resistant strains. The high incidence of *tet*(M) among our tetracycline-resistant isolates is in agreement with the wide distribution of this gene among *Lactobacillus* spp. isolated from fermented dry sausages (Gevers et al. 2003a) and in DNA extracted directly from pork meat (Garofalo et al. 2007). These results indicate also that the spread of tetracycline resistance genes persists despite the ban of this antibiotic as a growth promoter in the European Union (Rizzotti et al. 2005).

The number of salami presenting erythromycin-resistant isolates was the same as for tetracycline (17 out of 20), but here the number of erythromycin resistant strains compared to the total number of strains was much lower, with only five out of 20 salami containing 100% of antibiotic resistant strains; in these salami, the microbiological counts were very low ($10^2 – 10^3$ cfu g^{-1}).

The genetic determinant for erythromycin resistance was found in all 17 salami containing resistant strains, and *erm*(B) was the most frequently detected gene, consistent with previous reports (Garofalo et al. 2007; Aquilanti et al. 2007). All 17 salami contained this gene, and *erm*(B) was detected in 83% of the erythromycin resistant strains. The *erm*(C) gene was only detected in two salami and in a small number of strains (two), confirming previous reports of lower prevalence (Aquilanti et al. 2007).

We identified 15 out of 20 salami as containing double-resistant strains, and of these, 14 carried both *erm* and *tet* genes and two carried two *tet* genes. Here, the microbiological counts were also low, with the exception of four salami that presented counts of 10^7 cfu g^{-1} (one containing erythromycin resistant strains and three with tetracycline resistant strains). No double resistant salami had high microbiological counts in response to both antibiotics. The simultaneous presence of *tet* and *erm* genes has been described in enterococci, streptococci, and staphylococci (Chopra and Roberts 2001; Rizzotti et al. 2005) and recently in lactobacilli (Huys et al. 2008). Moreover, the simultaneous occurrence of *tet*(M)/*tet*(W) and *tet*(M)/*tet*(S) is in agreement with reports of Gram-positive bacteria containing multiple *tet* genes that can have either the same mode of action (efflux or ribosomal protection) or different modes of action (efflux and ribosomal protection) (Chopra and Roberts 2001). Recently, Simeoni et al. (2008) found that 72.7% of staphylococci isolates carried two tetracycline resistance determinants, underscoring the great diffusion of this type of resistance.

Our study provides evidence of the occurrence of tetracycline and, to a lesser extent, of erythromycin resistant lactobacilli in fermented dry sausage produced in northern Italy. Although a low level of resistance in the intestinal flora of food animals should be thought of as a distinguishing safety mark for food animals (Van den Bogaard et al. 1997), in our study 80% of salami could be considered as safe even though 20% could represent a border line situation regarding the possibility of transferring AR genes to pathogens because only a high number (10^7-10^8 cfu g^{-1}) of cell donors had a detectable effect on the

number of the recipients and in this way it can be discriminated (Jacobsen et al. 2007).

Further investigations should be applied to other food production chains, food-associated bacteria, and to the possibility of AR gene transfer in order to evaluate the health risk of the presence of AR bacteria in foods.

A follow-up study within several years would be helpful in ascertaining whether the incidence of AR in fermented meat products decreases following the ban on the use of antibiotics as growth promoters in January 1, 2006 (Regulation (EC) n° 1831/2003).

Acknowledgements

This study was funded by the Ministero delle Politiche Agricole e Forestali, project "ARAFOA – Risk assessment related to the antibiotic resistance in bacteria used for the production of fermented food (cheese and cultured meat), especially with regard to typical and PDO products". Chapter 7303, D.M. 662/7303/03 dated 23/12/2003.

References

Aarestrup, F.M. (2005) Veterinary drug usage and antimicrobial resistance in bacteria of animal origin. *Basic Clin Pharmacol Toxicol* **96**, 271-81.

Aminov, R.I., Garrigues-Jeanjean, N., Mackie, R.I. (2001) Molecular ecology of tetracycline resistance: development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. *Appl Environ Microbiol* **67**, 22-32.

Aquilanti, L., Santarelli, S., Silvestri, G., Osimani, A., Petruzzelli, A. and Clementi, F. (2007) The microbial ecology of a typical Italian salami during its natural fermentation. *Int J Food Microbiol* **120**, 136-145.

Aymerich, T., Martín, B., Garriga, M., Vidal-Carou, M.C., Bover-Cid, S. and Hugas, M. (2006) Safety properties and molecular strain typing of lactic acid bacteria from slightly fermented sausages. *J Appl Microbiol* **100**, 40-49.

Chopra, I., Roberts, M. (2001) Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol Mol Biol Rev* **65**, 232-260.

Comi, G., Urso, R., Iacumin, L., Rantsiou, K., Cattaneo, P., Cantoni, C. and Cocolin, L. (2005) Characterisation of naturally fermented sausages produced in the North East of Italy. *Meat Sci* **69**, 381–392.

Coppola, S., Mauriello, G., Aponte, M., Moschetti, G. and Villani, F. (2000) Microbial succession during ripening of Naples-type salami, a southern Italian fermented product. *Meat Sci* **56**, 321-329.

Danielsen, M. and Wind, A. (2003) Susceptibility of *Lactobacillus* spp. to antimicrobial agents. *Int J Food Microbiol* **82**, 1–11.

EFSA (2005) Opinion of the Scientific Panel on additives and products or substances used in animal feed on the updating of the criteria used in the assessment of bacteria for resistance to antibiotics of human or veterinary importance. *The EFSA Journal* **223**, 1-12.

Egervärn, M., Danielsen, M., Roos, S., Lindmark, H. and Lindgren, S. (2007) Antibiotic susceptibility profiles of *Lactobacillus reuteri* and *Lactobacillus fermentum*. *J Food Prot* **70**, 412-418.

Flórez, A.B., Delgado, S. and Mayo, B. (2005) Antimicrobial susceptibility of lactic acid bacteria isolated from a cheese environment. *Can J Microbiol* **51**, 51–58.

Garofalo, C., Vignaroli, C., Zandri, G., Aquilani, L., Bordoni, D., Osimani, A., Clementi, F., Biavasco, F. (2007) Direct detection of antibiotic resistance genes in specimens of chicken and pork meat. *Int J Food Microbiol* **113**, 75-83.

Gevers, D., Huys, G. and Swings, J. (2001) Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. *FEMS Microbiol Lett* **205**, 31-36.

Gevers, D., Masco, L., Baert, L., Huys, G., Debevere, J. and Swings, J. (2003a) Prevalence and diversity of tetracycline resistant lactic acid bacteria and their *tet* genes along the process line of fermented dry sausages. *Syst Appl Microbiol* **26**, 277–283.

Gevers, D., Danielsen, M., Huys, G., Swings, J. (2003b) Molecular characterization of *tet*(M) genes in *Lactobacillus* isolates from

different types of fermented dry sausage. *Appl Environ Microbiol* **69**, 1270-1275.

Hammes, W.P., Knauf, H.J. (1994) Starters in processing of meat products. *Meat Sci* **36**, 155-168.

Heinemann, J.A., Ankenbauer, R.G. and Amábile-Cuevas, C.F. (2000) Do antibiotics maintain antibiotic resistance? *Drug Discov Today* **5**, 195-204.

Huys, G., D'Haene, K., Collard, J.M. and Swings, J. (2004) Prevalence and molecular characterization of tetracycline resistance in *Enterococcus* isolates from food. *Appl Environ Microbiol* **70**, 1555–1562.

Huys, G., D'Haene, K., Danielsen, M., Mättö, J., Egervärn, M. and Vandamme, P. (2008) Phenotypic and molecular assessment of antimicrobial resistance in *Lactobacillus paracasei* strains of food origin. *J Food Prot* **71**, 339-44.

Iacumin, L., Comi, G., Cantoni, C. and Cocolin, L. (2006) Ecology and dynamics of coagulase-negative cocci isolated from naturally fermented Italian sausages. *Syst Appl Microbiol* **29**, 480–486.

Jacobsen, L., Wilcks, A., Hammer, K., Huys, G., Gevers, D., Andersen, S.R. (2007) Horizontal transferof *tet*(M) and *erm*(B) resistance plasmids from food strains of *Lactobacillus plantarum* to *Enterococcus faecalis* JH2-2 in the gastrointestinal tract of gnotobiotic rats. *FEMS Microbiol Ecol* **59**, 158-166.

Jensen, L.B., Frimodt-Moller, N., Aarestrup, F.M. (1999) Presence of *erm* gene classes in Gram-positive bacteria of animal and human origin in Denmark. *FEMS Microbiol Lett* **170**, 151-158.

Leroy, F. and de Vuyst, L. (2004). Lactic acid bacteria as functional starter cultures for the food industry. *Trends Food Sci Technol* **15**, 67– 78.

Lucke, F.K. (1985) Fermented sausages. In *Microbiology of Fermented Foods* ed. Wood, B.J.B. pp. 41–83. London: Elsevier Applied Science.

Miralles, M.C., Flores, J. and Perez-Martinez, G. (1996) Biochemical tests for the selection of *Staphylococcus* strains as potential meat starter cultures. *Food Microbiol* **13**, 227–236.

Olsvik, B., Olsen, I., Tenover, F.C. (1995) Detection of *tet*(M) and *tet*(O) using the polymerase chain reaction in bacteria isolated from patients with periodontal disease. *Oral Microbiol Immunol* **10**, 87-92.

Ouoba, L.I.I., Lei, V., Jensen, L.B. (2008) Resistance of potential probiotic lactic acid bacteria and bifidobacteria of African and European origin to antimicrobials: Determination and transferability of the resistance genes to other bacteria. *Int J Food Microbiol* **121**, 217-224.

Parente, E., Greco, S., Crudele, M.A. (2001) Phenotypic diversity of lactic acid bacteria isolated from fermented sausages produced in Basilicata (Southern Italy). *J Appl Microbiol* **90**, 943-952.

Phillips, I., Casewell, M., Cox, T., De Groot, B., Friis, C., Jones, R., Nightingale, C., Preston, R., Waddell, J. (2004) Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. *J Antimicrob Chemother* **53**, 28-52.

Poyart, C., Jardy, L., Quesne, G., Berche, P., Trieu-Cuot, P. (2003) Genetic basis of antibiotic resistance in *Streptococcus agalactiae* strains isolated in a French hospital. *Antimicrob Agents Chemother* **47**, 794-797.

Rantsiou, K., Urso, R., Iacumin, L., Cantoni, C, Cattaneo, P., Comi, G. and Cocolin, L. (2005) Culture-dependent and -independent methods to investigate the microbial ecology of Italian fermented sausages. *Appl Environ Microbiol* **71**, 1977–1986.

Rantsiou, K. and Cocolin, L. (2006) New developments in the study of the microbiota of naturally fermented sausages as determined by molecular methods: a review. *Int J Food Microbiol* **108**, 255-67.

Rizzotti, L., Simeoni, D., Cocconcelli, P., Gazzola, S., Dellaglio, F., Torriani, S. (2005) Contribution of enterococci to the spread of antibiotic resistance in the production chain of swine meat commodities. *J Food Prot* **68**, 955-965.

Saarela, M., Maukonen, J., von Wright, A., Vilpponen-Salmela, T., Patterson, A.J., Scott, K.P., Hämynen, H., Mättö, J. (2007) Tetracycline susceptibility of the ingested *Lactobacillus acidophilus* LaCH-5 and *Bifidobacterium animalis* subsp. *lactis* Bb-12 strains during antibiotic/probiotic intervention. *Int J Antimicrob Agents* **29**, 271-280.

Simeoni, D., Rizzotti, L., Cocconcelli, P., Gazzola, S., Dellaglio, F., Torriani, S. (2008) Antibiotic resistance genes and identification of staphylococci collected from the production chain of swine meat commodities. *Food Microbiol* **25**, 196-201.

Sorensen, T.L., Blom, M., Monnet, D.L., Frimodt-Moller, N., Poulsen, R.L. and Espersen, F. (2001) Transient intestinal carriage after ingestion of antibiotic resistant *Enterococcus faecium* from chicken and pork. *New Engl J Med* **345**, 1161-1166.

Talon, R., Leroy, S., Lebert, I., Giammarinaro, P., Chacornac, J.P., Latorre-Moratalla, M., Vidal-Carou, C., Zanardi, E., Conter, M. and Lebecque, A. (2008) Safety improvement and preservation of typical

sensory qualities of traditional dry fermented sausages using autochthonous starter cultures. *Int J Food Microbiol* **126**, 227–234.

Teale, C.J. (2002) Antimicrobial resistance and the food chain. *J Appl Microbiol* **92**, 85S-89S.

Tenover, F.C. and Hughes, J.M. (1996) The challenges of emerging infectious diseases. Development and spread of multiply-resistant bacterial pathogens. *J Am Med Assoc* **275**, 300–304.

Teuber, M., Meile, L. and Schwarz, F. (1999) Acquired antibiotic resistance in lactic acid bacteria from food. *Antonie van Leeuwenhoek* **76**, 115–137.

Urso, R., Comi, G. and Cocolin, L. (2006) Ecology of lactic acid bacteria in Italian fermented sausages: isolation, identification and molecular characterization. *Syst Appl Microbiol* **29**, 671–680.

Van den Bogaard, A.E., Mertens, P., London, N.H., Stobberingh, E.E. (1997) High prevalence of colonization with vancomycin- and pristinamycinresistant enterococci in healthy humans and pigs in the Netherlands: is the addition of antibiotics to animal feeds to blame? *J Antimicrob Chemother* **40**, 454–456.

Ventura, M., Casas, I.A., Morelli, L. and Callegari, M.L. (2000) Rapid amplified ribosomal DNA restriction analysis (ARDRA) identification of *Lactobacillus* spp. isolated from faecal and vaginal samples. *Syst Appl Microbiol* **23**, 504-509.

Villani, F., Casaburi, A., Pennacchia, C., Filosa, L., Russo, F. and Ercolini, D. (2007) Microbial ecology of the soppressata of Vallo di Diano, a traditional dry fermented sausage from southern Italy, and in vitro and in situ selection of autochthonous starter cultures. *Appl Environ Microbiol* **73**, 5453-5463.

641 **Table 1** Primer sequences, target genes, amplicon sizes, reference strains used as positive controls and PCR
642 protocol references used for the detection of selected AR genes
643

Primer pair	Target gene	Sequence (5'–3')	Amplicon size (bp)	Reference	Positive control strains
erm(B)-1	*erm*(B)	GGTAAAGGGCATTTAACGAC	454	Poyart et al. 2003	*L. sakei 13*⋆
erm(B)-2		CGATATTCTCGATTGACCCA			
erm(C)-1	*erm*(C)	ATCTTTGAAATCGGCTCAGG	294	Jensen et al. 1999	*L. reuteri* 70⋆
erm(C)-2		CAAACCCGTATTCCACGATT			
tet(M)-1	*tet*(M)	GAACTCGAACAAGAGGAAAGC	740	Olsvik et al. 1995	*L. plantarum* 30⋆
tet(M)-2		ATGGAAGCCCAGAAAGGAT			
tet(L)-FW	*tet*(L)	GTMGTTGCGCGCTATATTCC	696	Gevers et al. 2003b	*E. faecium* LMG20927†
tet(L)-RV		GTGAAMGRWAGCCCACCTAA			
tet(W)-FW	*tet*(W)	GAGAGCCTGCTATATGCCAGC	168	Aminov et al. 2001	*Bifidobacterium animalis* subsp. *lactis* Bb-12‡
tet(W)-RV		GGGCGTATCCACAATGTTAAC			
tet(S)-FW	*tet*(S)	GAAAGCTTACTATACAGTAGC	169	Aminov et al. 2001	*L. plantarum* 31⋆
tet(S)-RV		AGGAGTATCTACAATATTTAC			

644 ⋆ Collection of microorganisms of the Microbiology Institute, Università Cattolica del Sacro Cuore, Piacenza (Italy).
645 † BCCM/LMG, Bacteria Collection, Belgium
646 ‡ Saarela *et al* 2007
647
648
649
650
651
652

Table 2 MIC data for *Lactobacillus* species from fermented dry sausages determined by microdilution in LSM broth

Antimicrobial	Species (n° of isolates tested)	MIC range (µg/ml)	MIC50 (µg/ml)	MIC90 (µg/ml)
Tetracycline	*L. sakei* (24)	2-512	16	256
	L. curvatus (16)	2-512	16	256
	L. plantarum (12)	16-512	256	512
	L. paracasei (3)	2-512	32	512
	L. brevis (2)	16-512	16	512
	L. rhamnosus (2)	256	256	256
	L. reuteri (1)	512	512	512
Erythromycin	*L. sakei* (24)	0.25-256	4	32
	L. curvatus (16)	0.25-128	4	32
	L. plantarum (12)	0.25-512	4	128
	L. paracasei (3)	0.25-1024	512	1024
	L. brevis (2)	32-128	32	128
	L. rhamnosus (2)	4-32	4	32
	L. reuteri (1)	512	512	512

677 **Table 3** Antibiotic resistance and occurrence of AR genes among *Lactobacillus* species from fermented dry
678 sausages
679

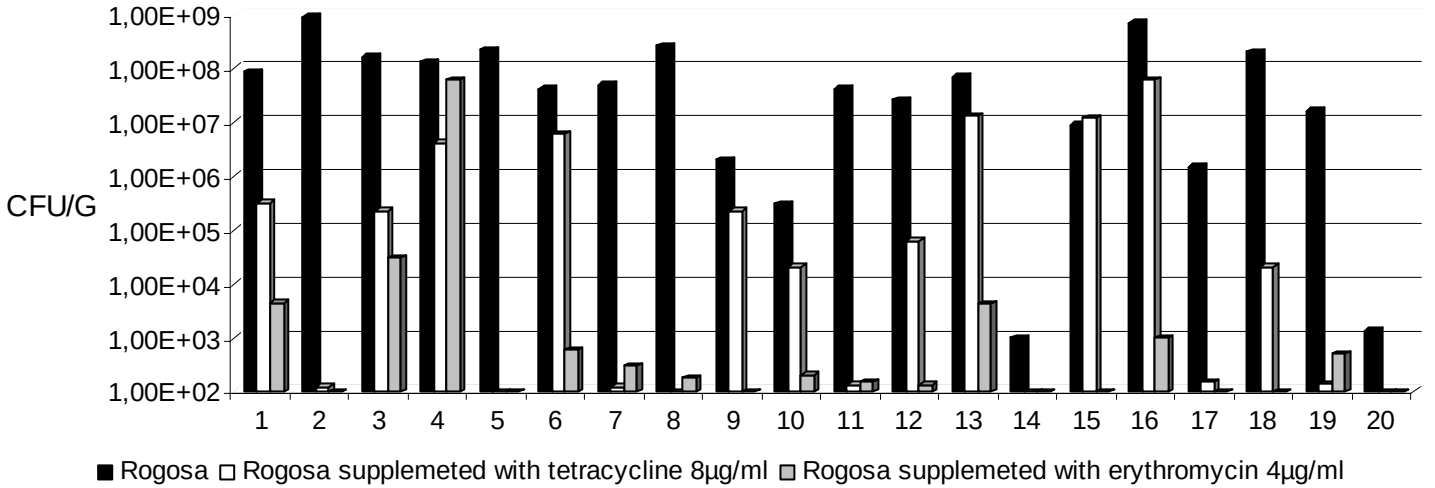
	Total strain number	Tetra cycline resistant strains	*tet*(M)	*tet*(W)	*tet*(S)	*tet*(L)	Erythro mycin resistant strains	*erm*(B)	*erm*(C)	Double resistant strains	*erm*+*tet* genes	Multiple *tet* genes
L. sakei	24	17	11	1	0	0	7	5	0	7	1	1
L. curvatus	16	10	7	4	0	0	10	10	0	8	11	4
L. plantarum	12	11	5	4	1	0	6	3	2	5	2	0
L. paracasei	3	2	2	0	0	0	2	1	0	2	0	0
L. brevis	2	2	1	0	0	0	2	2	0	2	1	0
L. rhamnosus	2	2	0	1	0	0	2	1	0	2	1	0
L. reuteri	1	1	1	0	0	0	1	1	0	1	0	0
Total	**60**	**45**	**27**	**10**	**1**	**0**	**30**	**23**	**2**	**27**	**16**	**5**

680
681
682
683
684
685
686
687
688
689
690
691
692

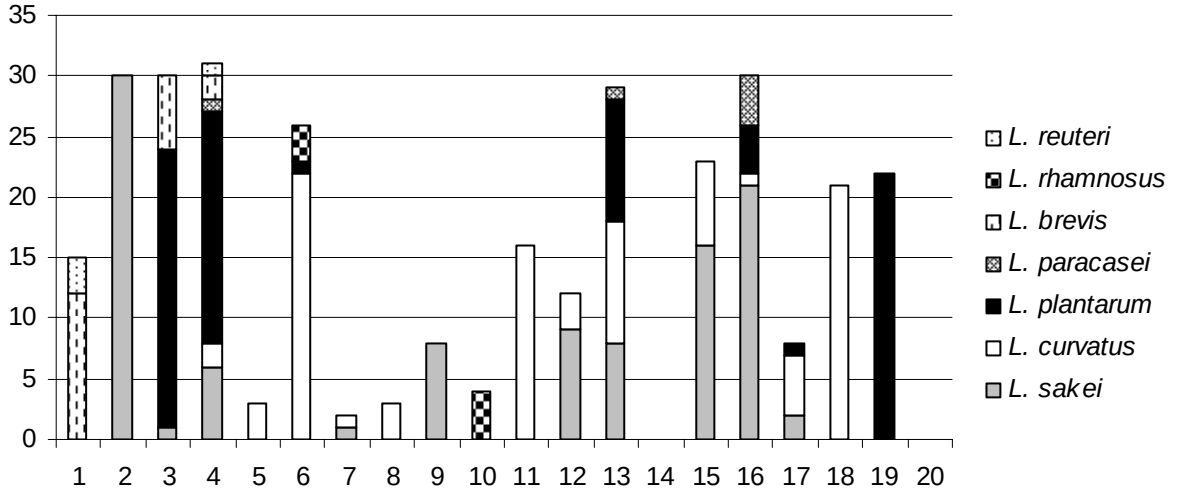
693 **Table 4** Antibiotic resistance, occurrence of AR genes and double resistance in 20 salami
694
695

Salami	Total strain number	Tetracycline resistant strains	*tet*(M)	*tet*(W)	*tet*(S)	*tet*(L)	Erythromycin resistant strains	*erm*(B)	*erm*(C)	Double resistant strains	*erm*+*tet* genes	Multiple *tet* genes
1	2	2	2	0	0	0	2	2	0	2	2	0
2	2	2	0	0	0	0	1	1	0	1	0	0
3	5	5	3	0	1	0	3	2	1	3	1	1
4	9	9	6	1	1	0	6	5	0	6	5	0
5	1	1	1	1	0	0	1	1	0	1	2	0
6	9	7	4	5	0	0	5	4	0	5	8	0
7	2	1	0	0	0	0	1	1	0	0	0	0
8	1	0	0	0	0	0	1	1	0	0	0	0
9	3	3	3	1	0	0	1	1	0	1	2	0
10	1	1	0	1	0	0	1	1	0	1	1	0
11	4	1	1	1	0	0	3	3	0	1	2	0
12	9	5	5	1	0	0	3	3	0	3	4	0
13	6	6	4	3	0	0	3	3	0	3	5	0
14	0	0	0	0	0	0	0	0	0	0	0	0
15	8	5	2	1	0	0	3	2	0	3	3	0
16	10	7	3	1	0	0	5	3	0	4	3	0
17	3	2	0	0	0	0	0	0	0	0	0	0
18	2	2	1	1	0	0	2	2	0	2	2	0
19	4	4	3	1	0	0	2	1	1	2	2	1
20	0	0	0	0	0	0	0	0	0	0	0	0
Total	**81**	**63**	**38**	**18**	**2**	**0**	**43**	**36**	**2**	**38**	**42**	**2**

Figure 1. Microbial counts of total and tetracycline- and erythromycin-resistant lactobacilli isolated from 20 Italian salami



704 **Figure 2.** Species diversity between tetracycline- and erythromycin-resistant lactobacilli (n=312) isolated from 20
705 Italian salami



706
707
708
709
710

CHAPTER 3

DISCUSSION AND CONCLUSION

In modern food animal production, antimicrobial agents have been used for therapy, as metaphylactis, prophylactis and as growth promoters (Aarestrup 2005). Therapeutic usage of antimicrobials in animals is important to prevent the epidemic spread of animal disease and to protect animal welfare. It can also prevent the transfer of zoonotic disease from animals to man (Ungemach 2000). However, the widespread and indiscriminate use of antibiotics in human and veterinary medicine and in livestock breeding has led to a spread of AR among both pathogenic and commensal microorganisms (Phillips et al., 2004).

In recent years, the food chain has been recognized as one of the main routes of transmission of AR from animal to human bacterial populations. In support of this, it has been demonstrated that the same type of genes encoding resistance to tetracycline and erythromycin have been found in commensal lactobacilli as well as in potentially pathogenic enterococci and streptococci (Teuber et al., 1999).

Most recent investigations in this regard have focused mainly on pathogenic bacteria (Gevers et al., 2003; Rizzotti et al., 2005), with fewer reports on AR lactobacilli available (Jacobsen et al., 2007). In view of that, the present study was planned to document the incidence of tetracycline and erythromycin resistant lactobacilli isolated from a food chain of a fermented dry sausage and from the end products obtained from artisanal factories producing Piacentino salami.

The presence of high lactobacilli counts in fermented dry sausages (about 10^7-10^8 cfu/g) observed in the present study is in agreement with the findings of other authors (Parente et al. 2001, Rantsiou et al. 2005, Aquilanti et al. 2007). In fact, LAB are usually present in raw meat at low numbers but they rapidly dominate fermentation due to the anaerobic environment

and the presence of nitrate and nitrite, conditions that favour their growth (Hammes and Knauf 1994).

It is important to notice that the antibiotic resistant species found in this study were the same found usually in fermented dry sausages. We confirmed, as has been reported, that *L. sakei*, *L. curvatus* and *L. plantarum* are the species most frequently recovered from meat products, especially in dry fermented sausages (Hugas et al. 1993, Gevers et al. 2000; Parente et al. 2001, Aymerich et al. 2003; Urso et al. 2006), with *L. sakei* being the most frequently isolated species in the production chain and in the end products. These species are known to be very well adapted to the specific conditions of fermented sausages (low pH and a_w) (Gevers et al. 2000).

In swine stool samples, *L. reuteri* was the prevalent species, as has been reported by previous studies, according to which *L. reuteri* is a common *Lactobacillus* species in pig intestine (Axelsson and Lindgren 1987; Pryde et al. 1999; Leser et al. 2002; Korhonen et al. 2007).

We isolated 312 *Lactobacillus* colonies from the end product (ascribed to 60 different strains) and 426 *Lactobacillus* colonies from the food chain, comprising 92 different strains (70 strains from the food chain and 22 from swine stools). Considering that in the production chain 10^5 cfu/g erythromycin-resistant lactobacilli were isolated from the casing but that there were less than 10^2 cfu/g isolated from the minced meat, we can speculate that part of bacteria that occurred during the ripening originated in the casing. Moreover, taking into account the REP-PCR fingerprinting, we can see that four different strains isolated in the casing were also found in different steps of the process line, and two of them were also found in the end of ripening (45 days). These same strains were found neither in the minced meat, nor in swine stools, suggesting that contamination occurred

between the casing and the food chain steps. To our knowledge this is the first study that demonstrates that the casing can represent a font of AR lactobacilli during the fermentation of a dry sausage.

Even though lactobacilli are generally susceptible to antibiotics inhibiting the synthesis of proteins, such as chloramphenicol, erythromycin, clindamycin and tetracycline, and more resistant to aminoglycosides (neomycin, kanamycin, streptomycin and gentamicin) (Charteris et al., 1998b; Coppola et al., 2005; Zhou et al., 2005), in this study we found high values of AR to erythromycin and specially to tetracycline. In fact, resistant strains to these agents have also been identified by other authors (Danielsen and Wind, 2003; Delgado et al., 2005; Florez et al., 2005).

The results of studies regarding the MIC of a certain strain differ according not only to their origin but also to the bacteria growth medium and the method used when testing the susceptibility to antimicrobials, and to the raw material used during the production when testing strains of food origin. Egervarn et al. (2007a) determinate the antibiotic susceptibility profiles of *L. reuteri* from different sources using the broth microdilution method and in this study he found that all 56 *L. reuteri* strains studied were resistant to tetracycline but only 6 of the 56 were resistant to erythromycin. A similar result was found by Korhonen et al. (2007) who studied by the plate dilution method faecal samples from healthy piglets and in this case 44 of 45 *L. reuteri* strains were resistant to tetracycline but none of them were resistant to erythromycin. Aymerich et al. (2006) studied the susceptibility to antibiotics based on the agar overlay disc diffusion test from LAB from slightly fermented sausages and in this case only 10.8% of *L. sakei* and 13.2% of *L. curvatus* were resistant to tetracycline and non of them were resistant to erythromycin. Aquilanti et al. (2007) documented the incidence

of resistance to various antibiotics in LAB isolated from swine and poultry meat samples by using the broth microdilution method and it has been observed that 2 out of 3 *L. plantarum* and the 2 *L. reuteri* analysed were tetracycline resistant but none of the 6 strains of *L. plantarum* and one of *L. reuteri* were erythromycin resistant. Danielsen and Wind (2003) analyzed with E-test 18 *L. plantarum* and 6 *L. sakei/curvatus* from a culture collection and none of them were erythromycin resistant and only one *L. plantarum* and one *L. sakei/curvatus* were tetracycline resistant. Flòrez et al. (2006) reported the MICs for 81 *L. plantarum* strains from different geographic locations and fermented products using the microdilution method and observed that 27 strains were tetracycline resistant and none of them were erythromycin resistant.

Compared to the number of resistant isolates found by other authors, our values are higher for both antibiotics. *L. reuteri* in swine stools presented 100% of strains resistant to tetracycline and 73% to erythromycin. *L. sakei*, *L. curvatus* and *L. plantarum* which were the species most frequently found in the end products presented respectively 71%, 63% and 92% of strains resistant to tetracycline and 29%, 63% and 50% of strains resistant to erythromycin. In the production chain, *L. plantarum* and *L. sakei* (the most common species) presented 73% and 100% of tetracycline resistant strains and 36% and 50% of erythromycin resistant strains.

The MICs for erythromycin in the production chain and in stools ranged between 0.25 and 512 µg/ml and between 0.25 and 1024 µg/ml in the end products, and resistance to this antibiotic was found in 59.1% of the food chain strains, 68.5% of stools strains and 50% of the strains isolated in the end products. Besides, MIC values for this antimicrobial were at least two fold dilution steps higher than the breakpoint values in 36%, 61% and

35% respectively for the production chain, stools and end product strains. The MICs for tetracycline in the production chain and in stools ranged between 16 and 512 µg/ml and between 2 and 512 µg/ml in the end products, and resistance to this antibiotic was found in 90.9% of the food chain strains, 98.5% of stools strains and 75% of the strains isolated in the end products. MIC values for this antimicrobial were at least two fold dilution steps higher than the breakpoint values in 40%, 97% and 50% respectively for the production chain, stools and end product strains.

In the 20 end products, even though we found antibiotic resistant strains, counts on erythromycin plates were particularly low, with 19 out of 20 salami presenting at maximum 10^4 cfu/g and only one presented a count of 10^7 cfu/g. In the latter salami we isolated nine strains, six of them were erythromycin resistant and all were tetracycline resistant. Regarding the tetracycline, counts of 15 out of 20 salami were at maximum 10^5 cfu/g and only three presented counts of 10^7 cfu/g, but all of them presenting a high proportion of tetracycline resistant strains (62%, 70% and 100%). Comparing these results to the high counts of lactobacilli on the medium without the addition of antibiotics (14 out of 20 salami presenting counts between 10^7-10^8cfu/g), we can speculate that most end products do not represent a real safety risk for the consumers and only four out of 20 salami could represent a border line situation regarding the risk of transferring AR genes to pathogens because only a high number (10^7-10^8 cfu/g) of cell donors had a detectable effect on the number of the recipients and in this way it can be discriminated (Jacobsen et al. 2007).

The situation in the end product from the food chain (dry sausage at 45 days of ripening) is different. In this product 12 strains were isolated, 10 were found to be tetracycline resistant and seven erythromycin resistant.

The point is that the counts on tetracycline plate were 10^6 cfu/g, which in theory does not represent a safety risk, but the erythromycin counts were about 10^7 cfu/g, which could be considered a border line situation. An important consideration regarding these data is that the salami sampled from the food chain was obtained from the same producing plant of one of the 20 end products taken in the retail market, but in this case the results were quite different, with tetracycline counts of 10^5 cfu/g and erythromycin counts of 10^3 cfu/g. These consistent differences demonstrate that there is a great variability not only between producers, but also inside a plant production. The variation of the presence of tetracycline LAB in different batches of a given fermented dry sausage was observed also by Gevers et al. (2000). Because of these differences it would be very interesting to follow other food chains from the same product repeatedly and from different producers in order to have sufficient data to estimate the situation of the AR in strains from this important source.

The PCR was applied to detect the most important genes coding for erythromycin and tetracycline resistances in *Lactobacillus* and it showed to be a consistent instrument for the detection of specific genes. Considering all strains analyzed, 81% and 90% respectively of tetracycline and erythromycin resistant strains gave amplification for at least one AR gene, indicating the great spread of these genes in the food production chain under investigation and in the end products.

Regarding the tetracycline resistance, the *tet*(M) gene showed the highest frequency in the end products and along the food chain (60% in both cases), while *tet*(W) was found mainly (65.2%) in swine stools samples. Concerning the erythromycin resistance, *erm*(B) gene showed the highest frequency in the end products (76.6%), in the food chain (71.4%) and in

stools samples (91.6%). In fact, the prevalence of this genetic determinant in LAB has been demonstrated by other authors (Garofalo et al. 2007; Aquilanti et al. 2007). Compared with the high incidence of *tet*(M), *tet*(W) and *erm*(B) determinants in our isolates, the detection of *tet*(S) and *erm*(C) genes was considered to be an occasional event. Unfortunately we cannot make specific correlations between the use of antibiotics and the detected resistances because we do not have information about the antimicrobial therapy that occurred in the swine plant breeding analyzed in this study.

Although antibiotics are of enormous value to combat infectious diseases, their efficacy is being threatened by microbial resistance. In fact, an increasing number of multi resistance strains displaying atypical resistance levels to tetracycline and erythromycin has been isolated (Ammor et al., 2007). In this study, the presence of phenotypic multi resistance was observed in 50% of the food chain strains, 67.1% of stools strains and 45% of the end products strains, and the occurrence of a *tet* and *erm* gene in combination was detected in 58%, 87% and 86% of the multi resistant strains from the food chain, stools and end products respectively; while the simultaneous occurrence of two *tet* genes was observed in 6.5% of the isolates. The simultaneous presence of *tet* and *erm* genes has been described in enterococci, streptococci, and staphylococci (Chopra and Roberts, 2001; Rizzotti et al., 2005). The presence of multiple *tet* genes was already found in individual Gram-positive isolates (Chopra and Roberts 2001; Villedieu et al., 2003; Simeoni et al., 2008).

This study suggests that food systems should be systematically monitored in terms of ARs, and it is very important that producers control not only the raw material used in the production but also the casing in the case of fermented sausages in order to improve the quality of these products.

Further investigations should be applied to other food production chains, to other food-associated bacteria, and to the possibility of transfer of AR genes in order to evaluate the health risk of the presence of AR in foods. Besides, it would be beneficial to perform a follow-up study within a few years to ascertain whether the incidence of AR in the food chain of fermented meat products decreases following the ban on the use of antibiotics as growth promoters that came into effect in January 1, 2006 (Regulation (EC) n° 1831/2003).

CHAPTER 4

REFERENCES

Aarestrup, F.M., Agerso, Y., Gerner-Smidt, P., Madsen, M., Jensen, L.B., 2000. Comparison of antimicrobial resistance phenotypes and resistance genes in *Enterococcus faecalis* and *Enterococcus faecium* from humans in the community, broilers, and pigs in Denmark. Diagnostic Microbiology and Infectious Disease 37, 127–137.

Aarestrup, F.M., 2005. Veterinary drug usage and antimicrobial resistance in bacteria of animal origin. Basic and Clinical Pharmacology and Toxicology 96(4), 271-281.

Adams, M.R., 1999. Safety of industrial lactic acid bacteria. Journal of Biotechnology 68, 171–178.

Ahn, C., Collins-Thompson, D., Duncan, C., Stiles, M.E., 1992. Mobilization and location of the genetic determinant of chloramphenicol resistance from *Lactobacillus plantarum* caTC2R. Plasmid 27, 169–176.

Axelsson, L., Lindgren, S., 1987. Characterization and DNA homology of *Lactobacillus* strain isolated from pig intestine. Journal of Applied Bacteriology 62, 433–440.

Al-Hamidi, A.A., 2004. Properties and applications of proteinaceous antibiotics produced by lactic acid bacteria: a review. Journal of Saudi Chemical Society 8, 35–46.

Ammor, M.S., Flòrez, A.B., Mayo, B, 2007. Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria. Food Microbiology 24, 559–570.

Ammor, M.S., Flórez, A.B., van Hoek, A.H., de Los Reyes-Gavilán, C.G., Aarts, H.J., Margolles, A., Mayo, B., 2008. Molecular characterization of intrinsic and acquired antibiotic resistance in lactic acid bacteria and bifidobacteria. Journal of Molecular Microbiology and Biotechnology 14(1-

3), 6-15.

Andoh, A., Fujiyama, Y., 2006. Therapeutic approaches targeting intestinal microflora in inflammatory bowel disease. World Journal of Gastroenterology 12(28), 4452–4460.

Aquilanti, L., Garofalo, C., Osimani, A., Silvestri, G., Vignaioli, C., Clementi, F., 2007. Isolation and molecular characterization of antibiotic-resistant lactic acid bacteria from poultry and swine meat products. Journal of Food Protection 70(3), 557-565.

Aymerich, M.T., Hugas, M., Monfort, J.M., 1998. Bacteriocinogenic lactic acid bacteria associated with meat products. Food Science and Technology International 4, 141–158.

Aymerich, T., Martín, B., Garriga, M., Hugas, M., 2003. Microbial quality and direct PCR identification of lactic acid bacteria and non-pathogenic staphylococci from artisanal low-acid sausages. Applied and Environmental Microbiology 69, 4583–4594.

Aymerich, T., Martín, B., Garriga, M., Vidal-Carou, M.C., Bover-Cid, S., Hugas, M., 2006. Safety properties and molecular strain typing of lactic acid bacteria from slightly fermented sausages. Journal of Applied Microbiology 100(1), 40-49.

Bernardeau, M., Guguen, M., Vernoux, J.P., 2006. Beneficial lactobacilli in food and feed: long-term use, biodiversity and proposals for specific and realistic safety assessments. FEMS Microbiology Reviews 30(4), 487-513.

Bernardeau, M., Vernoux, J.P., Henri-Dubernet, S., Guéguen, M., 2008. Safety assessment of dairy microorganisms: The *Lactobacillus* genus. International Journal of Food Microbiology 126(3), 278-285.

Borriello, S.P., Hammes, W.P., Holzapfel, W., Marteau, P., Schrezenmeir, J., Vaara, M, Valtonen, V., 2003. Safety of probiotics that contain lactobacilli or bifidobacteria. Clinical Infectious Diseases 36, 775–780.

Boyle, R.J., Tang, M.L., 2006. The role of probiotics in the management of allergic disease. Clinical and Experimental Allergy 36(5), 568–576.

Carr, F.J., Chill, D., Maida, N., 2002. The lactic acid bacteria: a literature survey. Critical Reviews in Microbiology 28(4), 281-370.

Cataloluk, O., Gogebakan, B., 2004. Presence of drug resistance in intestinal lactobacilli of dairy and human origin in Turkey. FEMS Microbiology Letters 236, 7–12.

Casaburi, A., Aristoy, M-C., Cavella, S., Monaco, R., Ercolini, D., Toldrà, F., Villani, F., 2007. Biochemical and sensory characteristics of traditional fermented sausages of Vallo di Diano (Southern Italy) as affected by the use of starter cultures Meat Science 76, 295–307.

Chamba, J.F., Irlinger, F., 2004. Secondary and adjunct cultures. In: Cheese: Chemistry, Physics and Microbiology, vol. 1. Elsevier, Amsterdam, the Netherlands, pp. 191–206.

Charteris, W.P., Kelly, P.M., Morelli, L., Collins, J.K., 1998. Antibiotic susceptibility of potentially probiotic *Lactobacillus* species. Journal of Food Protection 61, 1636–1643.

Chopra, I., Roberts, M., 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiology and Molecular Biology Reviews 65(2), 232-260.

Cocconcelli, P.S., Cattivelli, D., Gazzola, S., 2003. Gene transfer of

vancomycin and tetracycline resistances among *Enterococcus faecalis* during cheese and sausage fermentations. International Journal of Food Microbiology 88(2-3), 315-323.

Cocolin, L., Manzano, M., Cantoni, C., Comi, G., 2001. Denaturing gradient gel electrophoresis analysis of the 16S rRNA gene V1 region to monitor dynamic changes in the bacterial population during fermentation of Italian sausages. Applied and Environmental Microbiology 67, 5113–5121.

Coenye, T., Vandamme, P., 2003. Extracting phylogenetic information from whole-genome sequencing projects: the lactic acid bacteria as a test case. Microbiology 149, 3507–3517.

Commission of European Communities, 2000. White paper on food safety. Commission of European Communities, Brussels, Belgium.

Coppola, S., Mauriello, G., Aponte, M., Moschetti, G., Villani, F., 2000. Microbial succession during ripening of Naples-type salami, a southern Italian fermented sausage Meat Science 56, 321-329.

Council of the European Communities, 1993. Directive 93/43/CEE, relative to the hygiene of the food products. OJEC L175, 1–11.

Cross, M.L., 2002. Microbes versus microbes: immune signals generated by probiotic lactobacilli and their role in protection against microbial pathogens. FEMS Immunology and Medical Microbiology 34, 245–253.

Danielsen, M., Wind, A., 2003. Susceptibility of *Lactobacillus* spp. to antimicrobial agents. International Journal of Food Microbiology 82, 1–11.

Davies, J., 1997. Origins, acquisition and dissemination of antibiotic resistance determinants. In: Antibiotic Resistance: Origins, Evolution,

Selection and Spread, vol. 207. Eds. Chadwick, D.J. and Goode, J. Ciba Foundation Symposium. Wiley, Chichester, pp. 15– 27.

Davison, J., 1999. Genetic exchange between bacteria in the environment. Plasmid 42, 73– 91.

Delgado, S., Flòrez, A.B., Mayo, B., 2005. Antibiotic susceptibility of *Lactobacillus* and *Bifidobacterium* species from the human gastrointestinal tract. Current Microbiology. 50, 202–207.

Donabedian, S.M., Thal, L.A., Hershberger, E., Perri, M.B., Chow, J.W., Bartlett, P., Jones, R., Joyce, K., Rossiter, S., Gay, K., Johnson, J., Mackinson, C., Debess, E., Madden, J., Angulo, F., Zervos, M.J., 2003. Molecular characterization of gentamicin-resistant enterococci in the United States: evidence of spread from animals to humans through food. Journal of Clinical Microbiology 41, 1109–1113.

Egervärn, M., Danielsen, M., Roos, S., Lindmark, H., Lindgren, S., 2007a. Antibiotic susceptibility profiles of *Lactobacillus reuteri* and *Lactobacillus fermentum*. Journal of Food Protection 70(2), 412-418.

Egervärn, M., Lindmark, H., Roos, S., Huys, G., Lindgren, S., 2007b. Effects of inoculum size and incubation time on broth microdilution susceptibility testing of lactic acid bacteria. Antimicrobial Agents And Chemotherapy 51(1), 394–396.

European Commission, 2005. Opinion of the FEEDAP Panel on the updating of the criteria used in the assessment of bacteria for resistance to antibiotics of human or veterinary importance. The EFSA Journal 223, 1–12.

Falagas, M.E., Betsi, G.I., Tokas, T., Athanasiou, S., 2006. Probiotics for prevention of recurrent urinary tract infections in women: a

review of the evidence from microbiological and clinical studies. Drugs 66 (9), 1253–1261.

Flórez, A.B., Delgado, S., Mayo, B., 2005. Antimicrobial susceptibility of lactic acid bacteria isolated from a cheese environment. Canadian Journal of Microbiology 51, 51–58.

Flórez, A.B., Egervärn, M., Danielsen, M., Tosi, L., Morelli, L., Lindgren, S., Mayo, B., 2006. Susceptibility of *Lactobacillus plantarum* strains to six antibiotics and definition of new susceptibility-resistance cutoff values. Microbial Drug Resistance 12(4), 252-256.

Franz, C.M.A.P., Stiles, M.E., Schleifer, K.H., Holzapfel, W.H., 2003. Enterococci in food—a conundrum for food safety. International Journal of Food Microbiology 88, 105–122.

Fontana, C., Cocconcelli, P.S., Vignolo, G., 2005. Monitoring the bacterial population dynamics during fermentation of artisanal Argentinean sausages International Journal of Food Microbiology 103, 131– 142.

Garofalo, C., Vignaroli, C., Zandri, G., Aquilanti, L., Bordoni, D., Osimani, A., Clementi, F., Biavasco, F., 2007. Direct detection of antibiotic resistance genes in specimens of chicken and pork meat. International Journal of Food Microbiology 113(1), 75-83.

Gevers, D., Huys, G., Devlieghere, F., Uyttendaele, M., Debevere, J., Swings, J., 2000. Isolation and identification of tetracycline resistant lactic acid bacteria from pre-packed sliced meat products. Systematic and Applied Microbiology 23(2), 279-284.

Gevers, D., Huys, G., Swings, J., 2003a. In vitro conjugal transfer of tetracycline resistance from *Lactobacillus* isolates to other Gram-positive bacteria. FEMS Microbiology Letters 225, 125-130.

Gevers, D., Masco, L., Baert, L., Huys, G., Debevere, J., Swings, J., 2003b. Prevalence and diversity of tetracycline resistant lactic acid bacteria and their *tet* genes along the process line of fermented dry sausages. Systematic and Applied Microbiology 26(2), 277-283.

Giraffa, G., 2002. Enterococci from foods. FEMS Microbiology Reviews 26, 163–171.

Hammes, W.P., Knauf, H.J., 1994. Starters in processing of meat products. Meat Science 36, 155-168.

Holzapfel, W.H., 2002. Appropriate starter culture technologies for small-scale fermentation in developing countries. International Journal of Food Microbiology 75, 197–212.

Hugas, M., Pagès, F., Garriga, M., Monfort, J.M., 1998. Application of the bacteriocinogenic *Lactobacillus sakei* CTC494 to prevent growth of Listeria in fresh and cooked meat products packaged with different atmospheres. Food Microbiology 15, 639–650.

Hugas, M., Monfort, J.M., 1997. Bacterial starter cultures for meat fermentation. Food Chemistry 59, 547– 554.

Hughes, V.M., Datta, N., 1983. Conjugative plasmids in bacteria of the dpre-antibioticT era. Nature 302, 725–726.

Huys, G., D'Haene, K., Swings, J., 2002. Influence of the culture medium on antibiotic susceptibility testing of food-associated lactic acid bacteria with the agar overlay disc diffusion method. Letters in Applied Microbiology 34, 402–406.

Huys, G., D'Haene, K., Swings, J., 2006. Genetic basis of tetracycline and minocycline resistance in potentially probiotic *Lactobacillus plantarum* strain CCUG 43738. Antimicrobial Agents and

Chemotherapy 50(4), 1550-1551.

Jacobsen, L., Wilcks, A., Hammer, K., Huys, G., Gevers, D., Andersen, S.R., 2007. Horizontal transfer of *tet(M)* and *erm(B)* resistance plasmids from food strains of *Lactobacillus plantarum* to *Enterococcus faecalis* JH2-2 in the gastrointestinal tract of gnobiotic rats. FEMS Microbiology Ecology 59, 158–166.

Klare, I., Konstabel, C., Müller-Bertling, S., Reissbrodt, R., Huys, G., Vancanneyt, M., Swings, J., Goossens, H., Witte, W., 2005. Evaluation of new broth media for microdilution antibiotic susceptibility testing of lactobacilli, lactococci, pediococci, and bifidobacteria. Applied and Environmental Microbiology 71, 8982–8986.

Klare, I., Konstabel, C., Werner, G., Huys, G., Vankerckhoven, V., Kahlmeter, G., Hildebrandt, B., Müller-Bertling, S., Witte, W., Goossens, H., 2007. Antimicrobial susceptibilities of *Lactobacillus*, *Pediococcus* and *Lactococcus* human isolates and cultures intended for probiotic or nutritional use. Journal of Antimicrobial Chemotherapy 59(5), 900-912.

Konigs, W.N., Kok, J., Kuipers, O.P., Poolman, B., 2000. Lactic acid bacteria: the bug of the new millennium. Current Opinion in Microbiology 3, 276–282.

Korhonen, J.M., Sclivagnotis, Y., von Wright, A.J., 2007. Characterization of dominant cultivable lactobacilli and their antibiotic resistance profiles from faecal samples of weaning piglets. Journal of Applied Microbiology 103, 2496-2503.

Kruse, H., Sorum, H., 1994. Transfer of multiple drug resistance plasmids between bacteria of diverse origins in natural microenvironments. Applied and Environmental Microbiology 60, 4015–4021.

Leser, T.D., Amenuvor, J.Z., Jensen, T.K., Lindecrona, R.H., Boye, M., Moller, K., 2002. Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. Applied and Environmental Microbiology 68, 673–690.

Leroy, F., De Vuyst, L., 2004. Lactic acid bacteria as functional starter cultures for the food industry. Trends in Food Science and Technology 15, 67– 78.

Levri, K.M., Ketvertis, K., Deramo, M., Merenstein, J.H., D'Amico, F., 2005. Do probiotics reduce adult lactose intolerance? A systematic review. Journal of Family Practice 54, 613–620.

Levy, S.B., 1997. Antibiotic resistance: an ecological imbalance. In: Antibiotic Resistance. Origins, Evolution, Selection and Spread. Eds. Chadwick, D.J. and Good, J. John Wiley and Sons, Chichester, pp. 1– 14.

Levy, S.B., Marshall, B., 2004. Antibacterial resistance world wide: causes, challenges and responses. Nature Medicine Supplement 10, S122–S129.

Lewis, G., 2006. Probiotics: a better way to treat infections during pregnancy. Midwifery Today with International Midwife 79, 30–31.

Lin, C.F., Fung, Z.F., Wu, C.L., Chung, T.C., 1996. Molecular characterization of a plasmid-borne (pTC82) chloramphenicol resistance determinant (cat-TC) from *Lactobacillus reuteri* G4. Plasmid 36, 116–124.

Ljungh, A., Wadstrom, T., 2006. Lactic acid bacteria as probiotics. Current Issues in Intestinal Microbiology 7(2), 73–89.

Lucke, F.K., 1985. Fermented sausages. In: Microbiology of Fermented Foods, vol. 2. Ed. Wood, B.J.B. London: Elsevier Applied Science, pp. 41–83.

Mathur, S., Singh, R., 2005. Antibiotic resistance in food lactic acid bacteria—a review. International Journal of Food Microbiology 105 (3), 281–295.

Matto, J., Suihko, M.L., Saarela, M., 2006. Comparison of three test media for antimicrobial susceptibility testing of bifidobacteria using the E-test method. International Journal of Antimicrobial Agents 28, 42–48.

McFarland, L.V., 2006. Meta-analysis of probiotics for the prevention of antibiotic associated diarrhoea and the treatment of *Clostridium difficile* disease. American Journal of Gastroenterology 101(4), 812–822.

Merk, K., Borelli, C., Korting, H.C., 2005. Lactobacilli – bacteria host interactions with special regard to the urogenital tract. International Journal of Medical Microbiology 295: 9–18.

Mevius, D.J., Sprenger, M.J.W., Wegener, H.C., 1999. Report EU conference "The Microbial Threat". International Journal of Antimicrobial Agents 11, 101–105.

Molly, K., Demeyer, D., Civera, T., Verplaetse, A., 1996. Lipolysis in a Belgian sausage: relative importance of endogenous and bacterial enzymes. Meat Science 43, 235–244.

Montel, M.C., Masson, F., Talon, R., 1998. Bacterial rule in flavour development. Proceedings, 44th International Congress on Meat Science and Technology, Barcelona, vol. 1, pp. 224–233.

Morot-Bizot, S.C., Leroy, S., Talon, R., 2006. Staphylococcal community of a small unit manufacturing traditional dry fermented sausages. International Journal of Food Microbiology 108: 210–217.

Normark, B.H., Normark, S., 2002. Evolution and spread of

antibiotic resistance. Journal of Internal Medicine. 252, 91–106.

Parente, E., Greco, S., Crudele, M.A., 2001. Phenotypic diversity of lactic acid bacteria isolated from fermented sausages produced in Basilicata (Southern Italy). Journal of Applied Microbiology 90: 943-952.

Perreten, V., Vorlet-Fawer, L., Slickers, P., Ehricht, R., Kuhnert, P., Frey, J., 2005. Microarray-based detection of 90 antibiotic resistance genes of Gram-positive bacteria. Journal of Clinical Microbiology 43, 2291–2302.

Phillips, I., Casewell, M., Cox, T., De Groot, B., Friis, C., Jones, R., Nightingale, C., Preston, R., Waddell, J., 2004. Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. of Antimicrobial Chemotherapy 53, 28–52.

Poole, K., 2002. Mechanisms of bacterial biocide and antibiotic resistance. Journal of Applied Microbiology 92, 55S– 64S.

Pryde, S.E., Richardson, A.J., Stewart, C.S., Flint, H.J., 1999. Molecular analysis of the microbial diversity present in the colonic wall, colonic lumen and cecal lumen of a pig. Applied and Environmental Microbiology 65, 5372–5377.

Rantsiou, K., Drosinos, E.H., Gialitaki, M., Urso, R., Krommer, J., Gasparik- Reichardt, J., Tòth, S., Metaxopoulos, I., Comi, G., Cocolin, L., 2005. Molecular characterization of *Lactobacillus* species isolated from naturally fermented sausages produced in Greece, Hungary and Italy. Food Microbiology 22, 19– 28.

Rantsiou, K, Cocolin, L., 2006. New developments in the study of the microbiota of naturally fermented sausages as determined by molecular methods: a review. International Journal of Food Microbiology 25;108(2):255-267.

Rizzotti, L., Simeoni, D., Cocconcelli, P.S., Gazzola, S., Dell'aglio, F., Torrioni, S., 2005. Contribution of enterococci to the spread of antibiotic resistance in the production chain of swine meat commodities. Journal of Food Protection 68(5):955-965.

Salminen, S., von Wright, A., Morelli, L., Marteau, P., Brassart, D., de Vos, W.M., Fondén, R., Saxelin, M., Collins, K., Mogensen, G., Birkeland, S.E., Mattila-Sandholm, T., 1998. Demonstration of safety of probiotics -- a review. International Journal of Food Microbiology 44(1-2), 93-106.

Salyers, A.A., Gupta, A., Wang, Y., 2004. Human intestinal bacteria as reservoirs for antibiotic resistance genes. Trends in Microbiology 12, 412–416.

Santos, E.M., Gonzalez-Fernandez, C., Jaime, I., Rovira, J., 1998. Comparative study of lactic acid bacteria house flora isolated in different varieties of chorizo. International Journal of Food Microbiology 39, 123–128.

Simeoni, D., Rizzotti, L., Cocconcelli, P.S., Gazzola, S., Dellaglio, F., Torriani, S., 2008. Antibiotic resistance genes and identification of staphylococci collected from the production chain of swine meat commodities. Food Microbiology 25(1), 196-201.

Sorensen, T.L., Blom, M., Monnet, D.L., Frimodt-Moller, N., Poulsen, R.L., Espersen, F., 2001. Transient intestinal carriage after ingestion of antibiotic resistant *Enterococcus faecium* from chicken and pork. New England Journal of Medicine 345, 1161–1166.

Sorum, H., L'Abée-Lund, T.M., 2002. Antibiotic resistance in food-related bacteria—a result of interfering with the global web of bacterial

genetics. International Journal of Food Microbiology 78, 43–56.

Sundsfjord, A., Simonsen, G.S., Courvalin, P., 2001. Human infections caused by glycopeptide-resistant *Enterococcus* spp: are they a zoonosis? Clinical Microbiology and Infection 7, 16–33.

Swenson, J.M., Ferraro, M.J., Sahm, D.F., Charache, P., Tenover, F.C., 1992. New vancomycin disk diffusion breakpoints for enterococci. The National Committee for Clinical Laboratory Standards Working Group on Enterococci. Journal of Clinical Microbiology 30, 2525–2528.

Strommenger, B., Kettlitz, C., Werner, G., Witte, W., 2003. Multiplex PCR assay for simultaneous detection of nine clinically relevant antibiotic resistance genes in *Staphylococcus aureus*. Journal of Clinical Microbiology 41, 4089–4094.

Talon, R., Leroy, S., Lebert, I., 2007. Microbial ecosystems of traditional fermented meat products: The importance of indigenous starters. Meat Science 77, 55–62.

Teale, C.J., 2002. Antimicrobial resistance and the food chain. Journal of Applied Microbiology 92, 85S-89S.

Tenover, F.C., Hughes, J.M., 1996. The challenges of emerging infectious diseases. Development and spread of multiply-resistant bacterial pathogens. Journal of the American Medical Association 275, 300-304.

Teuber, M., Meile, L., Schwarz, F., 1999. Acquired antibiotic resistance in lactic acid bacteria from food. Antonie van Leeuwenhoek 76, 115–137.

Teuber, M., 1999. Spread of antibiotic resistance with food-borne pathogens. Cellular and Molecular Life Sciences 56, 755– 763.

Teuber, M., Perreten, V., 2000. Role of milk and meat products as

vehicles for antibiotic-resistant bacteria. Acta Veterinaria Scandinavica Supplementum 93, 75– 87.

Teuber, M., 2001. Veterinary use and antibiotic resistance. Current Opinion in Microbiology 4, 493–499.

Tollefson, L., Angulo, F.J., Fedorka-Cray, P.J., 1998. National surveillance for antibiotic resistance in zoonotic enteric pathogens. Veterinary Clinics of North America: Food Animal Practice 14, 141– 150.

Torres, C., Rojo-Bezares, B., Sàenz, Y., Zarazaga, M., Ruiz-Larrea, F., 2005. Antibiotic resistance phenotypes and mechanisms of resistance in lactic acid bacteria of oenological origin. Eighth Symposium on Lactic Acid Bacteria: Genetics, metabolism, and applications. FEMS, Egmond aan Zee, The Netherlands.

Torriani, S., Dell'aglio, F., Palummeri, M., 1990. Characterization of lactobacilli isolated from Italian salami. Annals of Microbiology, 40:225–233.

Turnidge, J., 2004. Antibiotic use in animals—prejudices, perceptions and realities. Journal of Antimicrobial Chemotherapy 53, 26–27.

Ungemach, F.R., 2000. Figures on quantities of antibacterials used for different purposes in the EU countries and interpretation. Acta Veterinaria Scandinavica Supplementum 93, 89–98.

Villani, F., Casaburi, A., Pennacchia, C., Filosa, L., Russo, F., Ercolini, D., 2007. Microbial ecology of the soppressata of Vallo di Diano, a traditional dry fermented sausage from southern Italy, and in vitro and in situ selection of autochthonous starter cultures. Applied and Environmental Microbiology.;73(17):5453-63.

Villedieu, A., Dìaz-Torres, M.L., Hunt, N., McNab, R., Spratt, D.A., Wilson, M., Mullany, P., 2003. Prevalence of tetracycline resistance genes in oral bacteria. Antimicrobial Agents and Chemotherapy 47, 878–882.

Volkmann, H., Schwartz, T., Bischoff, P., Kirchen, S., Obst, U., 2004. Detection of clinically relevant antibiotic-resistance genes in municipal wastewater using real-time PCR (TaqMan). Journal of Microbiolical Methods 56, 277–286.

Young, P., Cash, B.D., 2006. Probiotic use in irritable bowel syndrome. Current Gastroenterology Reports 8 (4), 321–326.

Zhou, J.S., Pillidge, C.J., Gopal, P.K., Gill, H.S., 2005. Antibiotic susceptibility profiles of new probiotic *Lactobacillus* and *Bifidobacterium* strains. International Journal of Food Microbiology 98, 211–217.